The role of hypothalamic mTORC1 and adipose tissue mTORC2 in organismal energetics

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Verena Maria Albert
aus Basel, Basel-Stadt

Basel, 2015

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Dr. Michael N. Hall und Prof. Dr. Philipp E. Scherer

Basel, den 23.06.2015

Prof. Dr. Jörg Schibler, Dekan

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 3.0 Schweiz
(CC BY-NC-ND 3.0 CH)

Sie dürfen: **Teilen** — den Inhalt kopieren, verbreiten und zugänglich machen

Unter den folgenden Bedingungen:

Namensnennung — Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen.

Keine kommerzielle Nutzung — Sie dürfen diesen Inhalt nicht für kommerzielle Zwecke nutzen.

Keine Bearbeitung erlaubt — Sie dürfen diesen Inhalt nicht bearbeiten, abwandeln oder in anderer Weise verändern.

Wobei gilt:

- **Verzichtserklärung —** Jede der vorgenannten Bedingungen kann **aufgehoben** werden, sofern Sie die ausdrückliche Einwilligung des Rechteinhabers dazu erhalten.
- **Public Domain (gemeinfreie oder nicht-schützbare Inhalte) —** Soweit das Werk, der Inhalt oder irgendein Teil davon zur Public Domain der jeweiligen Rechtsordnung gehört, wird dieser Status von der Lizenz in keiner Weise berührt.
- **Sonstige Rechte —** Die Lizenz hat keinerlei Einfluss auf die folgenden Rechte:
    - Die Rechte, die jedermann wegen der Schranken des Urheberrechts oder aufgrund gesetzlicher Erlaubnisse zustehen (in einigen Ländern als grundsätzliche Doktrin des **fair use** bekannt);
    - Die **Persönlichkeitsrechte** des Urhebers;
    - Rechte anderer Personen, entweder am Lizenzgegenstand selber oder bezüglich seiner Verwendung, zum Beispiel für **Werbung** oder Privatsphärenschutz.
- **Hinweis —** Bei jeder Nutzung oder Verbreitung müssen Sie anderen alle Lizenzbedingungen mitteilen, die für diesen Inhalt gelten. Am einfachsten ist es, an entsprechender Stelle einen Link auf diese Seite einzubinden.

Quelle: http://creativecommons.org/licenses/by-nc-nd/3.0/ch/ Datum: 12.11.2013

Table of contents

Table of contents (continued)

I. Summary

Mammalian Target of Rapamycin (mTOR) signaling is a crucial regulator of cell growth and metabolism. The highly conserved serine/threonine protein kinase mTOR is activated by growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), nutrients, and it is sensitive to the cellular energy state. mTOR forms two structurally and functionally distinct multi-protein complexes, termed mTOR complex 1 (mTORC1) and mTORC2, through which it regulates distinct downstream processes, such as protein synthesis, lipogenesis, nucleotide biosynthesis, glucose metabolism, autophagy and cell survival. Deregulation of mTOR signaling is often associated with metabolic diseases, such as diabetes, obesity and cancer. While the role of mTOR signaling in regulating growth and metabolism in single cells is quite well understood, the non-cell autonomous effects of mTOR signaling are still only poorly characterized.

In order to better understand the role of tissue-specific mTOR signaling in organismal energetics, we investigated the role of mTORC1 signaling in hypothalamic orexigenic Agrp neurons and its influence on systemic energy homeostasis and feeding behavior. We therefore generated Agrp neuron-specific *raptor* knockout (Agrp-*raptor* KO) mice, which display inactive mTORC1 signaling in Agrp neurons. Agrp-*raptor* KO mice exhibited a decrease in the circadian expression of orexigenic neuropeptides, but they did not show any defects in energy homeostasis and feeding behavior when fed either a standard diet or a high fat diet. Thus, our findings demonstrate that mTORC1 signaling in Agrp neurons is dispensable for the regulation of systemic energy homeostasis and feeding behavior.

In the second part of this thesis, we investigated the role of mTORC2 signaling in adipose tissue with particular focus on how adipose mTORC2 affects non-shivering thermogenesis (NST) and cold-induced glucose uptake. We found that mTORC2 signaling was induced in brown adipocytes by β-adrenergic stimulation via cAMP, Epac1 and PI3K. Furthermore, mTORC2 signaling in adipose tissue was required for temperature homeostasis, since mice lacking mTORC2 signaling in mature adipocytes (adipose tissue specific

rictor knockout (AdRiKO) mice) were hypothermic and sensitive to cold stress. While lipid store mobilization and induction of oxidative metabolism and mitochondrial uncoupling were not impaired in AdRiKO mice, inactivation of mTORC2 signaling in adipose tissue resulted in a significant impairment in cold-induced glucose uptake and glycolysis in brown adipose tissue (BAT). Interestingly, restoration of glucose metabolism in BAT via introduction of a constitutively active form of Akt2 or via over-expression of hexokinase II increased body temperature and improved cold tolerance of AdRiKO mice. Hence, our findings identify mTORC2 in BAT as a novel regulator of systemic energy homeostasis upon NST by affecting cold-induced glucose uptake and glycolysis.

Taken together, this thesis provides new insights into the non-cell autonomous functions of mTORC1 in Agrp neurons and mTORC2 in BAT. These findings could facilitate the development of novel drugs to treat metabolic disorders, such as obesity and diabetes.

II. Abbreviations

4E-BP	eukaryotic translation initiation factor (eIF4E) binding protein
AdRaKO	adipose tissue-specific raptor knockout
AdRiKO	adipose tissue-specific rictor knockout
Agrp	Agouti-related peptide
Agrp-*raptor* KO	Agrp neuron-specific raptor knockout
Agrp-*rictor* KO	Agrp neuron-specific rictor knockout
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
AS160	Akt substrate of 160kDa
ATG13	autophagy-related 13
ATG14	autophagy-related 14
ATGL	adipose tissue triglyceride (TG) lipase
BAD	bcl-2 associated death promoter
BAT	brown adipose tissue
Bim	bcl-2-like 11
BNIP3	bcl-2/adenovirus E1B 19kDa interacting protein
brite	brown in white
C/EBPα	CCAAT/enhancer binding protein α
C/EBPβ	CCAAT/enhancer binding protein β
C/EBPγ	CCAAT/enhancer binding protein γ
CAD	Carbamoyl phosphate synthethase 2, aspartate transcarbamoylase, and dihydroorotase
cAMP	cyclic AMP
CBP80	nuclear cap-binding protein subunit 1
ChIP	chromatin immunoprecipitation
CNS	central nervous system
DIO	diet-induced obesity
Dio2	type II iodothyronine deiodinase
DMH	dorsomedial hypothalamic nucleus
ECAR	extracellular acidification rate
Ednra	endothelin receptor type A

eEF2K	eukaryotic elongation factor 2 kinase
eIF4B	eukaryotic translation initiation factor 4B
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	eukaryotic translation initiation factor 4G
ETC	electron transport chain
FAs	fatty acids
FAT	focal adhesion kinase targeting
FATC	FRAP, ATP, TRAP C-terminal
FGF-21	fibroblast growth factor 21
FKBP	FK506 binding protein of 12kDa
FOX	forkhead box
FOXO	O-subfamily of the FOX proteins
FRB	FKBP12-rapamycin binding
G6P	glucose-6-phosphate
GFP	green fluorescent protein
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
GSK3	glycogen synthase kinase 3
HFD	high fat diet
HSL	hormone sensitive lipase
IGF-1	insulin-like growth factor 1
IKKα	IκB kinase α
IR	insulin receptor
IRS-1	insulin receptor substrate 1
JAKs	janus kinases
L-*raptor* KO	liver-specific raptor knockout
L-*TSC1* KO	liver-specific TSC1 knockout
LHA	lateral hypothalamic area
LiRiKO	liver-specific rictor knockout
LR	leptin receptor
MAG	monoglyceride lipase
MC-3R	melanocortin 3 receptor
MC-4R	melanocortin 4 receptor

MCP-1	monocyte-chemoattractant protein 1
mLST8	mammalian lethal with sec-13 protein
mSIN1	mammalian stress-activated map kinase-interacting protein 1
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
Myf5	myogenic factor 5
NE	norepinephrine
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NPY	neuropeptide Y
NST	non-shivering thermogenesis
OCR	oxygen consumption rate
PDK1	phosphoinositide-dependent kinase 1
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphatidylinositol 3-kinase-related kinase
PKA	protein kinase A
PKC	protein kinase C
POMC	pro-opiomelanocortin
POMC-*rictor* KO	POMC neuron-specific rictor knockout
POMC-*TSC1* KO	POMC neuron-specific TSC1 knockout
PPARα	peroxisome proliferator-activated receptor α
PPARγ	peroxisome proliferator-activated receptor γ
PPP	pentose phosphate pathway
PRDM16	PRD1-BF-1-RIZ1 homologous domain-containing protein-16
Psat	phosphoserine aminotransferase
PTEN	phosphatase and tensin homolog
PVN	paraventricular nucleus
RAG	RAS-related GTP-binding protein
RAmKO	raptor muscle knockout
raptor	regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
rictor	rapamycin insensitive companion of mTOR

RImKO	rictor muscle knockout
S6K	S6 kinase
SGK	serum- and glucocorticoid-induced protein kinase 1
SKAR	Aly/REF-like substrate
SREBP	sterol regulatory element-binding protein
STAT3	signal transducer and activator of transcription 3
sWAT	subcutaneous white adipose tissue
TBC1D7	TBC1 domain family member 7
TBP	TATA box binding protein
TFEB	transcription factor EB
TGs	triglycerides
TNF-α	tumor necrosis factor α
TOR	target of rapamycin
TSC1	tuberous sclerosis complex 1
TSC2	tuberous sclerosis complex 2
TSCmKO	TSC1 mucle knockout
UCP1	uncoupling protein 1
ULK1	UNC-51-like kinase 1
VO_2	oxygen consumption
WAT	white adipose tissue
ZT	Zeitgeber
α-MSH	α-melanocyte stimulating hormone

Chapter 1: Introduction

1. Introduction

1.1 The TOR signaling pathway

TOR – a key regulator of cell growth and metabolism

Target of Rapamycin (TOR) is a highly conserved serine/threonine kinase that is a key regulator of cell growth and metabolism. TOR has been identified in all eukaryotes examined to date, including worms, flies, mammals, and plants. TOR was originally discovered in yeast through isolation of yeast mutants, which were resistant to the growth-inhibitory effects of the macrolide rapamycin **(Heitman et al. 1991, Kunz et al. 1993)**. Before the discovery of TOR, cell growth was thought to be a passive, non-regulated process that occurred when nutrients were abundant. However, since the discovery of TOR, it has become evident that cell growth is a tightly regulated process that involves a complicated signaling network in order to adapt growth and metabolism to environmental conditions. TOR is a nutrient and energy sensor and forms the central node of a complex signaling network that senses the energy status of a cell or an organism and adapts growth and metabolism accordingly **(Wullschleger et al. 2006, Loewith and Hall 2011, Laplante and Sabatini 2012, Dibble and Manning 2013, Shimobayashi and Hall 2014)**.

Structure and complex formation of TOR

TOR is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family. While PI3Ks are lipid kinases, to date no lipid kinase activity has been observed for any member of the PIKK family **(Keith and Schreiber 1995)**. TOR and related PIKK members are thus exclusively protein kinases.

The mammalian TOR (mTOR) protein consists of 2549 amino acids and contains a number of distinct and highly conserved domains **(Figure 1)**. The N-terminal part of TOR consists of tandem HEAT repeats that are predicted to form a superhelical structure, which

enables interaction of TOR with other proteins **(Perry and Kleckner 2003)**. The C-terminal part of TOR contains a focal adhesion kinase targeting (FAT) domain and an FK506 binding protein of 12kDa (FKBP12)-rapamycin binding (FRB) domain. Rapamycin forms a complex with FKBP12, which binds to the FRB domain and inhibits TOR activity **(Brown et al. 1994, Sabatini et al. 1994, Stan et al. 1994)**. The FRB domain is followed by the highly conserved kinase domain and a FRAP, ATP, TRAP C-terminal (FATC) domain. All members of the PIKK family possess a FAT and FATC domain. While the exact role of this domain is yet unknown, it is speculated that it might be involved in the regulation of kinase activity **(Sekulic et al. 2000)**.

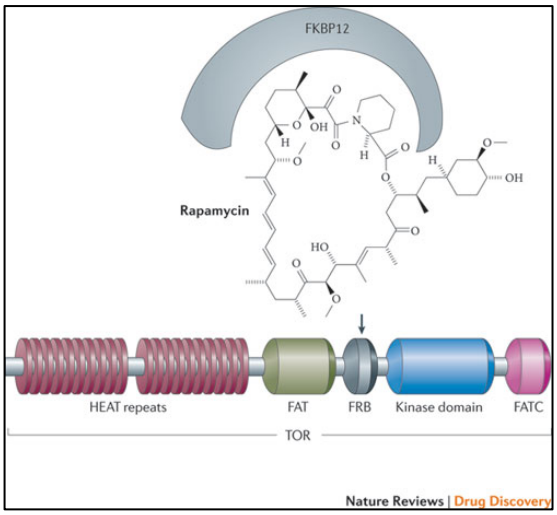


Figure 1. Structure of TOR **(Benjamin et al. 2011)**. See text for details.

TOR forms two structurally and functionally distinct complexes, which are highly conserved between eukaryotes. In mammals, these two complexes are called mammalian TOR Complex 1 (mTORC1) and mTORC2. mTORC1 consists of mTOR, mammalian lethal with sec-13 protein (mLST8), and regulatory associated protein of mTOR (raptor) **(Figure 2)**. mTORC2 contains mTOR, mLST8, mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and rapamycin insensitive companion of mTOR (rictor) **(Wullschleger et al. 2006, Loewith and Hall 2011, Laplante and Sabatini 2012, Dibble and Manning 2013, Shimobayashi and Hall 2014) (Figure 2)**. Importantly, while mTORC1 is sensitive to rapamycin, mTORC2 is rapamycin-insensitive since the FRB domain is masked when mTOR is in complex with the proteins forming the mTORC2 complex **(Jacinto et al. 2004, Sarbassov et al. 2004)**. In particular, in yeast it has suggested that the C-terminal part of the rictor ortholog Avo3 might be involved in

masking the FRB domain **(Gaubitz et al. 2015)**. Due to the rapamycin insensitivity of mTORC2 many other mTOR inhibitors have been developed, such as Torin1, Torin2, PP242, INK128, and AZD-8055, to name a few. These inhibitors block mTOR kinase activity by acting as ATP-competitive mTOR inhibitors and thus block both mTORC1 and mTORC2 activity **(Benjamin et al. 2011, Schenone et al. 2011)**. However, to date no mTORC2-specific inhibitor is available.

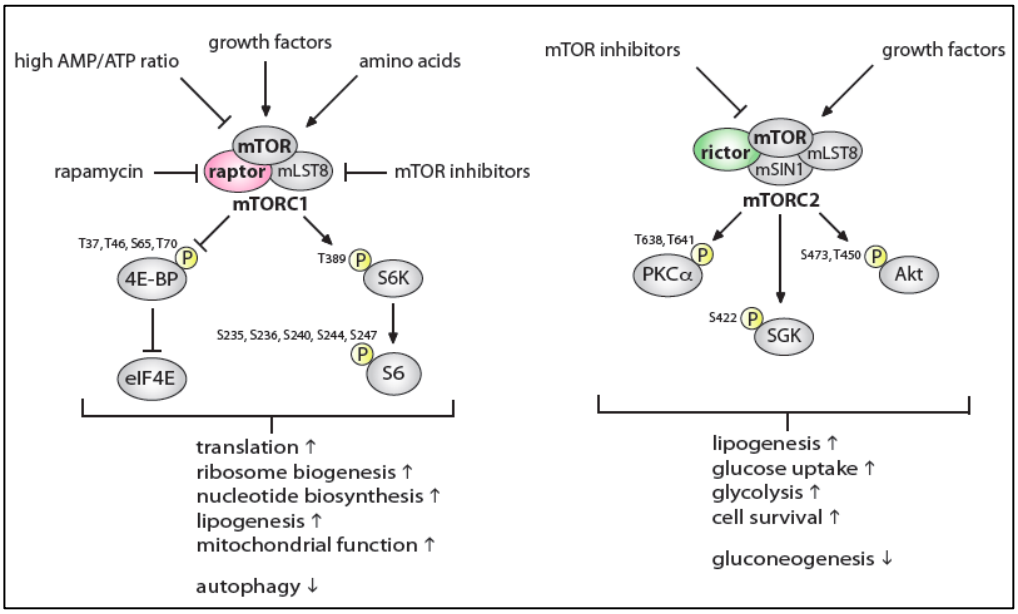


Figure 2. Overview on mTORC1 and mTORC2 signaling. See text for details.

Upstream of mTORC1 and mTORC2

mTORC1 and mTORC2 are activated by overlapping as well as distinct upstream signals and regulate different sets of downstream effector pathways **(Figure 2)**.

Upstream of mTORC1

mTORC1 is activated by growth factors, such as insulin or insulin-like growth factor 1 (IGF-1), amino acids and cellular energy **(Dibble and Manning 2013)**.

Growth factors. Growth factors activate mTORC1 via the PI3K/Phosphoinositide-dependent kinase 1 (PDK1)/Akt signaling pathway **(Figure 3)**. Once growth factors, such as insulin or IGF-1 bind to the insulin or IGF-1 receptor, this leads to phosphorylation and

activation of Akt via PI3K-mediated activation PDK1 **(Taniguchi et al. 2006, Laviola et al. 2007)**. Akt then inactivates the Tuberous Sclerosis Complex 1 (TSC1)-TSC2-TBC1 domain family member 7 (TBC1D7) complex by phosphorylating TSC2 at multiple sites. This activates mTORC1, since the TSC1-TSC2-TBC1D7 complex is a negative regulator of the small GTPase Ras homolog enriched in brain (Rheb) **(Inoki et al. 2002)**. Active Rheb in turn binds and activates mTOR via a still unknown mechanism **(Long et al. 2005)**.

Amino acids. Besides growth factors, mTORC1 is also activated by amino acids **(Figure 3)**. Amino acids activate mTORC1 in a RAS-related GTP-binding protein (RAG)-dependent manner **(Kim et al. 2008, Sancak et al. 2008, Sancak et al. 2010)**. There are four different RAG proteins, RAGA, RAGB, RAGC, and RAGD and they belong to a family of small GTPases. RAGs form heterodimers, where RAGA or RAGB forms a heterodimer with RAGC or RAGD. Upon amino acid stimulation, the RAG heterodimer converts to its active state via a still not fully understood mechanism. In this activated state RAGA or RAGB is loaded with GTP and RAGC or RAGD is loaded with GDP. Activation of the RAG heterodimer leads to the translocation of mTORC1 to the lysosome where it is activated by Rheb, which mediates the growth factor stimulated activation of mTORC1. Hence, for full activation of mTORC1 both amino acids and growth factors are required.

Cellular energy. Cellular energy status is the third major input that regulates mTORC1 activity **(Figure 3)**. When intracellular energy levels drop, a high intracellular AMP/ATP ratio activates AMP-activated protein kinase (AMPK). AMPK phosphorylates TSC2, thereby stimulating the GAP activity of the TSC1-TSC2-TBC1D7 complex towards Rheb, resulting in inhibition of mTORC1 **(Corradetti et al. 2004, Inoki et al. 2006)**. Additionally, AMPK also phosphorylates raptor, leading to binding of raptor to 14-3-3 scaffolding proteins and thereby inhibition of mTORC1 **(Gwinn et al. 2008)**. Consequently, when intracellular ATP levels are high, AMPK becomes inactive and this releases the inhibition of mTORC1.

Upstream of mTORC2

In comparison to the upstream regulation of mTORC1, less is known about upstream regulators of mTORC2. To date the only known upstream regulators of mTORC2 are growth factors **(Frias et al. 2006, Yang et al. 2006, Garcia-Martinez and Alessi 2008)**. Growth factors activate mTORC2 via a different mechanism compared to the growth factor-mediated activation of mTORC1. Activation of mTORC2 by growth factors is induced by PI3K-dependent association of mTORC2 with ribosomes **(Zinzalla et al. 2011) (Figure 3)**. However, how PI3K mediates association of mTORC2 with ribosomes still has to be elucidated.

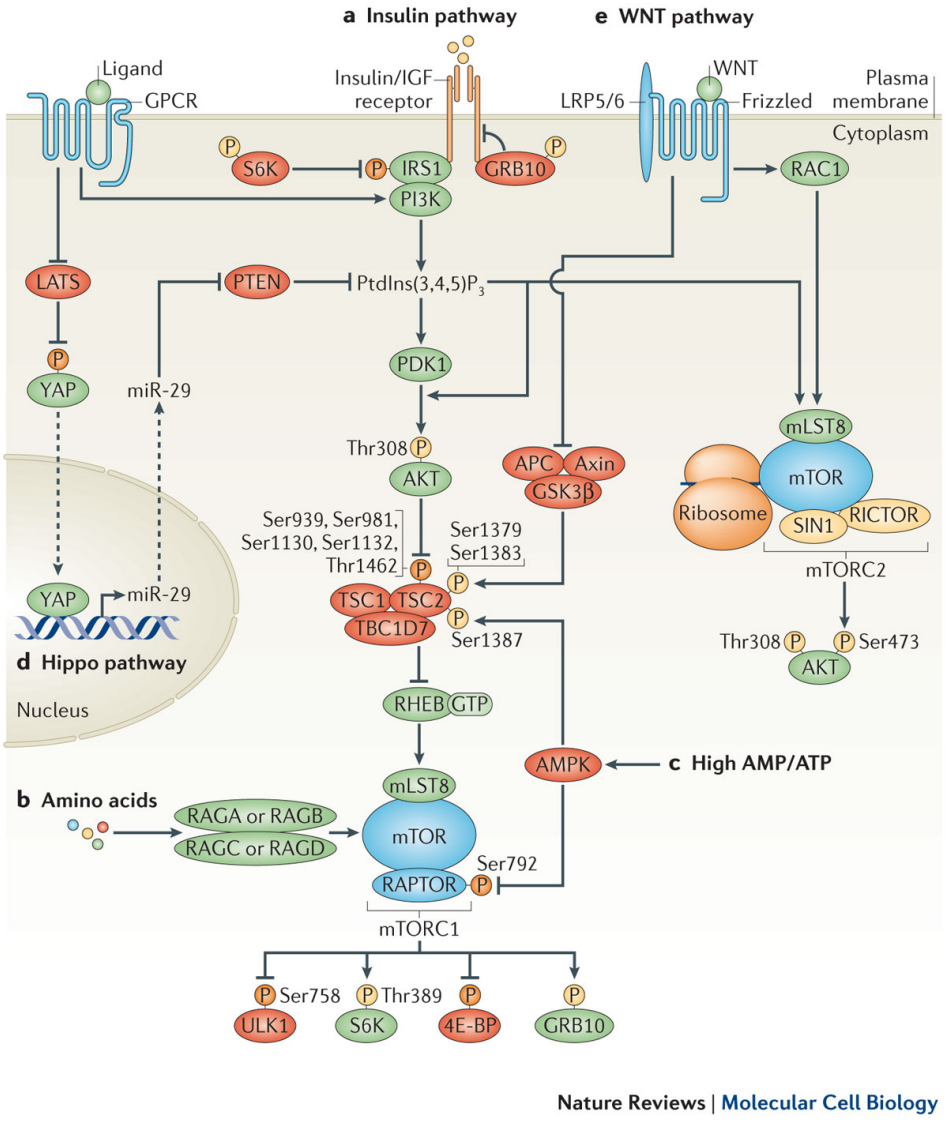


Figure 3. Upstream of mTOR signaling **(Shimobayashi and Hall 2014)**. See text for details.

Downstream of mTORC1 and mTORC2

mTORC1 and mTORC2 phosphorylate a distinct set of downstream targets through which they promote anabolic processes, such as protein synthesis, ribosome biogenesis, nucleotide biosynthesis, lipogenesis, cell survival and nutrient uptake, while they inhibit catabolic processes, such as autophagy **(Dibble and Manning 2013, Shimobayashi and Hall 2014) (Figure 2)**.

Downstream of mTORC1

The best characterized downstream targets of mTORC1 are ribosomal protein S6 kinase (S6K), eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs), and the autophagy activating kinase UNC-51-like kinase 1 (ULK1) **(Ma and Blenis 2009)**. In this section the main downstream processes regulated by mTORC1 will be discussed.

Protein synthesis. mTORC1 regulates protein synthesis mainly via S6K and 4E-BPs. Upon phosphorylation by mTORC1, 4E-BPs dissociate from eIF4E. This allows binding of eIF4G to eIF4E at the 5'-end of mRNAs, thereby promoting cap-dependent translation initiation. S6K in turn regulates protein synthesis through phosphorylation and activation of its downstream target S6, which stimulates expression of mRNAs involved in ribosome biogenesis **(Chauvin et al. 2014)**. Moreover, S6K also phosphorylates a number of proteins involved in translation or mRNA processing, such as eIF4B, eukaryotic elongation factor 2 kinase (eEF2K), programmed cell death 4 (PDCD4), nuclear cap-binding protein subunit 1 (CBP80), and S6K1 Aly/REF-like substrate (SKAR) **(Wilson et al. 2000, Wang et al. 2001, Raught et al. 2004, Richardson et al. 2004, Peschiaroli et al. 2006, Shahbazian et al. 2006)**. Hence, mTORC1 affects protein synthesis at multiple levels by regulating ribosome biogenesis protein expression, translation initiation, and mRNA processing.

Lipogenesis. Besides its role in stimulating protein synthesis, S6K is also involved in mediating the stimulatory effects of mTORC1 on lipogenesis **(Duvel et al. 2010)**. Transcription of lipogenic genes is

regulated by sterol regulatory element-binding protein (SREBP) transcription factors. mTORC1 stimulates lipogenesis by promoting SREBP maturation in an S6K-dependent fashion **(Duvel et al. 2010)**. Moreover, mTORC1 directly phosphorylates and inhibits phosphatidate phosphatase LPIN1 (Lipin-1), which is a negative regulator of SREBP **(Peterson et al. 2011)**.

Nucleotide biosynthesis. mTORC1 stimulates *de novo* nucleotide biosynthesis by promoting transcription of pentose phosphate pathway (PPP) genes **(Duvel et al. 2010)**. This increases the amount of intracellular ribose, which can serve as building blocks for purines and pyrimidines. Importantly, PPP gene transcription is dependent on SREBP. Thus, mTORC1 regulates PPP gene transcription in a similar fashion as lipogenic gene transcription **(Duvel et al. 2010)**. Additionally, mTORC1 stimulates CAD protein activity through S6K-mediated phosphorylation of CAD **(Ben-Sahra et al. 2013, Robitaille et al. 2013)**. CAD catalyzes the three initial rate-limiting steps of *de novo* pyrimidine synthesis, since it encodes three distinct enzymatic activities (carbamoyl phosphate synthethase 2, aspartate transcrabamoylase, and dihydroorotase).

Autophagy. Autophagy is a process where cellular components are degraded to remove damaged proteins and organelles and to provide substrates for energy production under nutrient-poor conditions. mTORC1 inhibits autophagy by directly phosphorylating several proteins involved in regulating autophagy. For example, mTORC1 phosphorylates ULK1, autophagy-related 13 (ATG13), and ATG14, leading to inhibition of autophagosome formation **(Ganley et al. 2009, Hosokawa et al. 2009, Jung et al. 2009, Kim et al. 2011)**. Moreover, mTORC1 also prevents transcription of lysosome biogenesis genes by phosphorylating transcription factor EB (TFEB). TFEB regulates transcription of genes involved in lysosome biogenesis and upon phosphorylation; TFEB is retained in the cytoplasm. This in turn blocks transcription of lysosome biogenesis genes when mTORC1 is active **(Roczniak-Ferguson et al. 2012)**.

Downstream of mTORC2

mTORC2 downstream targets are members of the AGC kinase family, such as Akt, serum/glucocorticoid regulated kinase (SGK) and protein kinase C (PKC) through which mTORC2 regulates lipogenesis, glucose uptake, glycolysis, and cell survival **(Sarbassov et al. 2004, Hresko and Mueckler 2005, Sarbassov et al. 2005, Facchinetti et al. 2008, Garcia-Martinez and Alessi 2008, Ikenoue et al. 2008, Jacinto and Lorberg 2008)**.

Lipogenesis. mTORC2 is a crucial regulator of lipogenesis in the liver. Hepatic mTORC2 promotes lipogenesis by stimulating SREBP1 expression and maturation in an Akt-dependent manner **(Hagiwara et al. 2012, Yuan et al. 2012)**. Hence, both mTORC1 and mTORC2 are important for the regulation of hepatic lipid homeostasis (see below for more details on the regulation of hepatic lipid metabolism by mTORC1 and mTORC2).

Glucose uptake and glycolysis. Upon insulin stimulation, glucose is taken up by extra-hepatic organs, such as skeletal muscle or adipose tissue and then degraded via glycolysis or stored in the form of glycogen. mTORC2 is a key regulator of glucose homeostasis by regulating insulin-stimulated glucose uptake, glycolysis and glycogenesis **(Kumar et al. 2008, Hagiwara et al. 2012)**. Insulin-stimulated glucose uptake is dependent on Akt-mediated phosphorylation of Akt substrate of 160kDa (AS160), which results in translocation of glucose transporter 4 (GLUT4) from internal vesicles to the plasma membrane **(Kane et al. 2002)**. Upon insulin stimulation, mTORC2 is required for activation of Akt and subsequent translocation of GLUT4 to the plasma membrane. Glycolytic flux is dependent on the rate of glucose uptake and on the activity of hexokinases, which phosphorylate glucose to generate glucose-6-phosphate (G6P). mTORC2 regulates glycolytic flux via Akt by promoting insulin-stimulated glucose uptake and by stimulating the expression of the liver-specific hexokinase 4 (also termed glucokinase) **(Kumar et al. 2008, Hagiwara et al. 2012)**. Finally, hepatic mTORC2 promotes

glycogenesis by inhibiting glycogen synthase kinase 3 (GSK3) in an Akt-dependent manner **(Hagiwara et al. 2012)**.

Cell survival. mTORC2 signaling plays a critical role in the promotion of cell survival via its downstream target Akt **(Manning and Cantley 2007)**. Akt phosphorylates and inhibits the pro-apoptotic protein bcl-2-associated death promoter (BAD), which blocks apoptosis **(Zha et al. 1996, Datta et al. 1997)**. Akt also phosphorylates IκB kinase α (IKKα), resulting in the activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling and the expression of pro-survival genes **(Ozes et al. 1999, Dan et al. 2008)**. Akt is a negative regulator of the O-subfamily of the forkhead box (FOX) transcription factor family (FOXO). Upon phosphorylation by Akt, FOXOs translocate from the nucleus to the cytoplasm, resulting in reduced transcription of FOXO-target genes **(Matsuzaki et al. 2003)**. FOXOs are important transcription factors involved in the regulation of apoptotic gene expression by inducing transcription of pro-apoptotic genes, such as bcl-2-like 11 (Bim) and bcl-2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) **(Dijkers et al. 2000, Dijkers et al. 2002, Stahl et al. 2002, Gilley et al. 2003)**. Hence, mTORC2 promotes cell survival by inhibiting the expression of genes involved in apoptosis via Akt-dependent inhibition of FOXO.

1.2 mTOR in metabolic organs

Due to the central role of mTOR in cell growth and metabolism, deregulation of the mTOR signaling pathway is often associated with the development of metabolic disorders, such as obesity, diabetes and cancer **(Dazert and Hall 2011, Laplante and Sabatini 2012)**. Hence, mTOR signaling in individual metabolic organs has a major impact on whole-body metabolism and energy homeostasis **(Table 1)**. To study *in vivo* functions of mTOR signaling in metabolic tissues, a large number of genetically modified mouse models with tissue-specific deletions of mTOR signaling components have been generated and characterized. In this section the phenotype of these mouse models

will be discussed and the role of mTORC1 and mTORC2 in liver, skeletal muscle, hypothalamus and adipose tissue will be described. An important point to consider when characterizing mice with chronic alterations of mTORC1 signaling is the negative feedback loop formed via S6K phosphorylation and inactivation of insulin receptor substrate 1 (IRS-1) **(Shah et al. 2004, Um et al. 2004, Ueno et al. 2005)**. Thus, induction of this feedback loop prevents activation of mTORC1 and mTORC2. Hence, in a state where mTORC1 signaling is chronically activated or inhibited this negative feedback loop causes alterations in mTORC2 and Akt signaling and could account for some of the observed phenotypes in these mouse models.

mTOR in liver

The liver is crucial for systemic metabolism by regulating glucose and lipid homeostasis, protein synthesis, detoxification of compounds, and hormone production. Analysis of genetically modified mice with liver-specific deletions of mTOR signaling components have revealed that the mTOR signaling pathway is important for liver function **(Table 1)**.

Hepatic mTORC1 is involved in the regulation of systemic glucose homeostasis **(Table 1)**. This has become evident since liver-specific *TSC1* knockout (L-*TSC1*-KO) mice with chronic mTORC1 activation in the liver are glucose intolerant **(Sengupta et al. 2010, Kenerson et al. 2011, Yecies et al. 2011)**. Hyperactivation of mTORC1 in the liver of L-*TSC1*-KO mice inhibits Akt signaling through constitutive activation of the negative feedback loop from S6K to IRS-1 **(Shah et al. 2004, Um et al. 2004, Ueno et al. 2005)**. Consequently, hepatic glucose uptake and glycolytic flux is reduced, resulting in impaired glucose clearance from the blood. In contrast, liver-specific *raptor* KO (L-*raptor*-KO) mice display increased hepatic Akt signaling, resulting in enhanced hepatic glucose uptake and improved systemic glucose tolerance **(Peterson et al. 2011, Umemura et al. 2014)**. Similar to mTORC1, hepatic mTORC2 also regulates systemic glucose homeostasis **(Table 1)**. In analogy with L-*TSC1*-KO mice, liver-specific *rictor* KO (LiRiKO) mice display impaired hepatic Akt signaling. This

results in a diabetic phenotype due to decreased insulin-stimulated hepatic glucose uptake and glycolysis **(Hagiwara et al. 2012)**.

Apart from its role in glucose metabolism, the liver is the major site of lipogenesis **(Eberle et al. 2004)**. As mentioned earlier, *in vitro* studies have shown that mTORC1 stimulates lipogenesis through induction of SREBP-1 expression, maturation and activation **(Porstmann et al. 2008, Duvel et al. 2010, Li et al. 2010, Peterson et al. 2011)**. Surprisingly, L-*TSC1*-KO mice display a decreased SREBP-1 activity and reduced lipogenesis despite hyper-activation of mTORC1 **(Sengupta et al. 2010, Kenerson et al. 2011, Yecies et al. 2011, Cornu et al. 2014)**. Thus, *in vivo* hyper-activation of mTORC1 is not sufficient to induce hepatic *de novo* lipogenesis. Similar to the regulation of glucose metabolism, there are also similarities between L-*TSC1*-KO mice and LiRiKO mice in regard to regulation of hepatic lipogenesis. Hepatic mTORC2 activates Akt and thereby stimulates SREBP-1 expression and maturation, which in turn activates lipogenesis **(Hagiwara et al. 2012)**. LiRiKO mice are thus hypolipidemic with a decrease in hepatic and circulating triglycerides due to impaired Akt-stimulated hepatic lipogenesis.

The liver is essential for the maintenance of whole-body energy homeostasis, since it converts excess nutrients, such as carbohydrates or lipids, to glycogen and triglycerides and stores them for periods of nutrient scarcity. During nutrient deprivation, the liver prevents systemic hypoglycemia through production of glucose via breakdown of glycogen (glycogenolysis) or via *de novo* synthesis of glucose (gluconeogenesis). Recently, it has become evident that hepatic mTORC1 signaling is crucial for the maintenance of whole-body energy homeostasis during nutrient deprivation **(Cornu et al. 2014)**. Chronic activation of mTORC1 in the liver upon fasting causes metabolic stress, which activates expression of the stress hormone fibroblast growth factor 21 (FGF-21). FGF-21 decreases locomotor activity and body temperature by acting on the central nervous system **(Bookout et al. 2013) (Table 1)**. Hence, proper regulation of hepatic mTORC1 activity

with regard to the energy status of an organism is crucial for maintenance of whole-body energy homeostasis.

mTOR in skeletal muscle

Skeletal muscle is the largest organ in the body, and modulation of skeletal muscle metabolism has substantial impact on whole-body energy homeostasis **(Egan and Zierath 2013)**. Characterization of mice with skeletal muscle-specific alterations in mTOR signaling has revealed that skeletal muscle mTOR signaling plays an important role in the regulation of systemic glucose metabolism, energy homeostasis, oxidative metabolism and skeletal muscle mass **(Table 1)**.

Upon insulin stimulation, skeletal muscle accounts for the majority of glucose removal from the circulation **(Yang 2014)**. Hence, skeletal muscle is crucial for the regulation of systemic glucose homeostasis. Skeletal muscle mTORC1 and mTORC2 are both involved in regulating systemic glucose homeostasis **(Table 1)**. Skeletal muscle-specific *rictor* KO (RImKO) mice display decreased Akt signaling in skeletal muscle, resulting in impaired insulin-stimulated glucose uptake and glycolysis in skeletal muscle and systemic glucose intolerance **(Kumar et al. 2008) (Table 1)**. Hence, skeletal muscle mTORC2 mediates insulin-induced glucose uptake and glycolysis. In contrast to this, skeletal muscle-specific *raptor* KO (RAmKO) mice display hyper-activated Akt in skeletal muscle due to lack of the negative feedback loop from S6K to IRS-1 **(Bentzinger et al. 2008)**. This results in increased glycogen accumulation in the muscles of RAmKO mice. However, despite enhanced Akt signaling in skeletal muscle, RAmKO mice are slightly glucose intolerant **(Bentzinger et al. 2008) (Table 1)**. Thus, inactivation of skeletal muscle mTORC1 exerts a negative effect on systemic glucose homeostasis, which cannot be reversed by hyper-activation of Akt.

Since mTORC1 is an important regulator of protein synthesis, skeletal muscle mTORC1 signaling is crucial for maintenance of skeletal muscle mass. In line with this, RAmKO mice display a progressive loss of muscle mass due to reduced rates of protein

synthesis **(Bentzinger et al. 2008) (Table 1)**. Surprisingly, skeletal muscle-specific *TSC1* KO (TSCmKO) mice are also dystrophic despite hyper-activation of mTORC1-stimulated protein synthesis **(Castets et al. 2013) (Table 1)**. The dystrophy in TSCmKO mice is due to impaired autophagy, which leads to muscle wasting due to accumulation of damaged proteins. Hence, mTORC1 activity in skeletal muscle must be tightly regulated, since both too much and too little mTORC1 activity leads to a deregulation of muscle homeostasis.

Apart from regulation of muscle mass and glucose homeostasis, mTORC1 signaling is also an important regulator of skeletal muscle oxidative function. Consequently, skeletal muscle of RAmKO mice displays a strong reduction in oxidative capacity **(Bentzinger et al. 2008) (Table 1)**. In contrast, hyperactivation of mTORC1 signaling in skeletal muscle in TSCmKO mice enhances oxidative activity, suggesting that mTORC1 stimulates oxidative function in skeletal muscle **(Bentzinger et al. 2013) (Table 1)**.

Finally, skeletal muscle mTORC1 signaling is important for the regulation of whole-body energy homeostasis. Both RAmKO and TSCmKO mice display a progressive loss of adipose tissue mass due to enhanced energy expenditure **(Bentzinger et al. 2008, Castets et al. 2013) (Table 1)**. Hence, similar to the effects on muscle mass, tight regulation of mTORC1 activity in skeletal muscle is crucial for the regulation of systemic energy homeostasis.

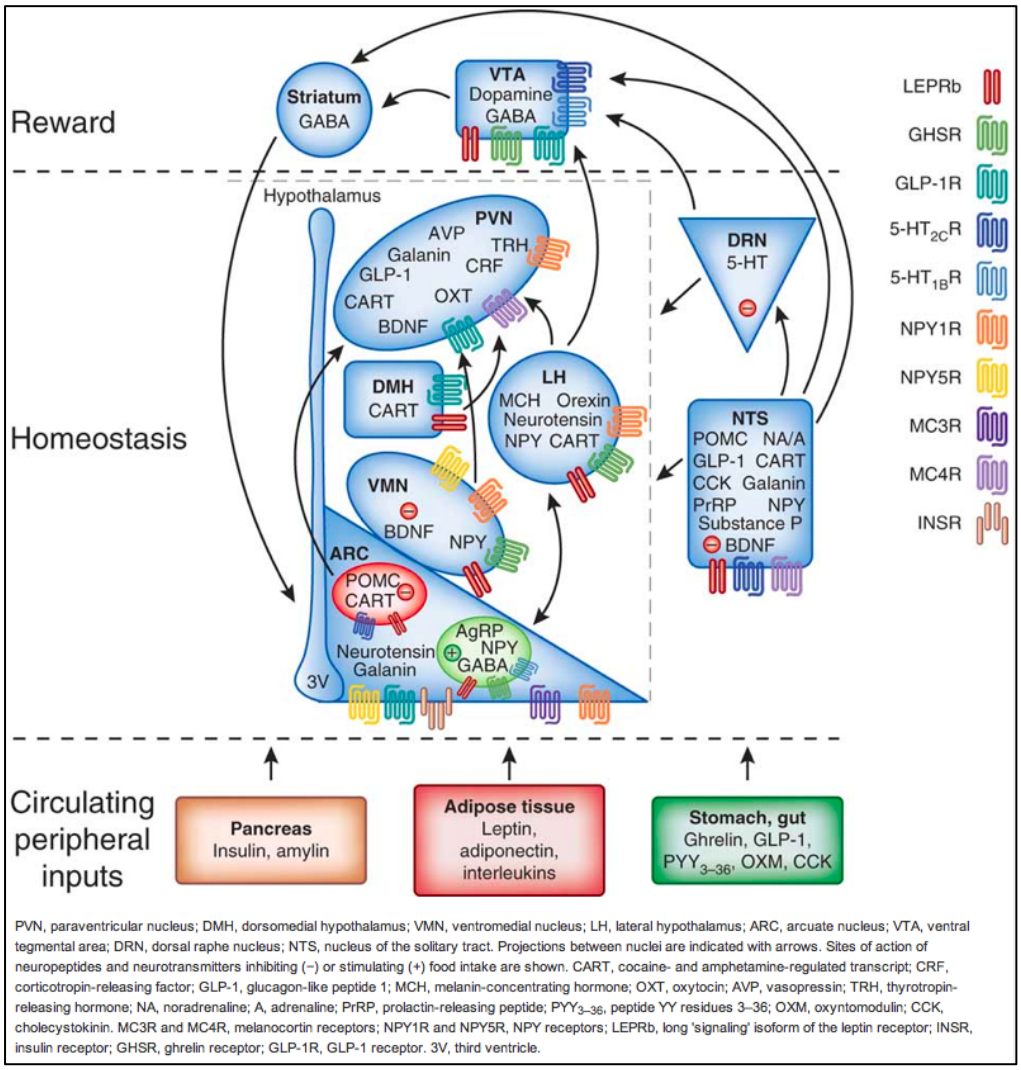
Organ with changed mTOR signaling	Effects on other organs or whole body	Mouse models
Adipose tissue/mature adipocytes		
mTORC1 inactivation	Energy expenditure ↑ Skeletal muscle insulin sensitivity ↑ Glucose tolerance ↑ Circulating leptin ↓ Locomotor activity ↓ Diet-induced obesity ↓	Adipose tissue-specific *raptor* KO mice **(Polak et al.)**
mTORC1 hyper-activation	Energy expenditure ↓ Thermogenesis ↓ Systemic insulin sensitivity ↓ Circulating insulin upon HFD feeding ↑ Glucose tolerance ↓	Adipose tissue-specific *Grb10* KO mice **(Liu et al.)**
mTORC2 inactivation	Body growth ↑ Systemic insulin sentitivity ↓ Glucose tolerance ↑ Circulating Insulin ↑ Hepatic steatosis ↑	Adipose tissue-specific *rictor* KO mice **(Cybulski et al., Kumar et al.)**
BAT/muscle progenitors		
mTORC2 inactivation	Energy expenditure ↑ Thermogenesis ↑ Hepatic steatosis ↓ Diet-induced obesity ↓ Glucose tolerance on HFD ↑	Myf5 positive BAT/muscle precursor cell-specific *rictor* KO mice **(Hung et al.)**
Liver/hepatocytes		
mTORC1 inactivation	Circulating cholesterol ↓ Glucose tolerance ↑	Liver-specific *raptor* KO mice **(Peterson et al., Umemura et al.)**
mTORC1 hyper-activation	Locomotor activity ↓ Body temperature ↓ Systemic glutamine depletion Glucose tolerance ↓ Circulating ketone bodies ↓	Liver-specific *TSC1* KO mice **(Cornu et al., Kenerson et al., Sengupta et al., Yecies at al.)**
mTORC2 inactivation	Systemic insulin sensitivity ↓ Circulating Insulin ↑ Glucose tolerance ↓ Circulating triglycerides ↓ Circulating cholesterol ↓ Male lifespan ↓	Liver-specific *rictor* KO mice **(Hagiwara et al., Yuan et al., Lamming et al. a, Lamming et al. b)**
Skeletal muscle		
mTORC1 inactivation	Adipose tissue mass ↓ Diet-induced obesity ↓ Glucose tolerance ↓ Lifespan ↓	Skeletal muscle-specific *raptor* KO mice **(Bentzinger et al.)**
mTORC1 hyper-activation	Adipose tissue mass ↓	Skeletal muscle-specific *TSC1* mice **(Castets et al.)**
mTORC2 inactivation	Glucose tolerance ↓	Skeletal muscle-specific *rictor* KO mice **(Kumar et al.)**
POMC neurons in the hypothalamus		
mTORC1 hyper-activation	Adipose tissue mass ↑ Circulating leptin ↑ Food intake ↑	POMC-specific *TSC1* KO mice **(Mori et al.)**
mTORC2 inactivation	Adipose tissue mass ↑ Glucose tolerance ↓ Food intake ↑	POMC-specific *rictor* KO mice **(Kocalis et al.)**
Agrp neurons in the hypothalamus		
mTORC2 inactivation	Glucose tolerance ↓	Agrp-specific *rictor* KO mice **(Kocalis et al.)**

Table 1. Effects of mTOR signaling in different organs on whole body metabolism **(Albert and Hall 2014)**. See text for details.

mTOR in hypothalamus

The central nervous system (CNS) and energy homeostasis

The CNS plays an essential role in the regulation of systemic energy homeostasis **(Luquet and Magnan 2009)**. The CNS senses the metabolic state of an organism by receiving humoral and neuronal signals from peripheral organs. Integration of these signals leads to adaptation of energy expenditure and feeding behavior in order to maintain systemic energy homeostasis. The importance of the CNS as a central regulator of energy homeostasis is underscored by the fact that individuals suffering from metabolic disorders often display a dysfunction in CNS-mediated control of energy homeostasis **(Velloso et al. 2009, Williams 2012)**. The brain region that is of particular importance for the regulation of systemic energy homeostasis is the hypothalamus **(Wilding 2002)**. The hypothalamus is localized above the brain stem and below the thalamus, forming the ventral part of the diencephalon. The hypothalamus contains several distinct regions that are involved in the regulation of feeding and energy expenditure **(Figure 4)**. These are the arcuate nucleus (ARC), the ventromedial nucleus of hypothalamus (VMH), the paraventricular nucleus (PVN), the dorsomedial hypothalamic nucleus (DMH), and the lateral hypothalamic area (LHA). Due to the proximity of the ARC to the median eminence it is viewed as a central integrator of hormonal signals originating from the periphery. At the median eminence-ARC contact site, the blood brain barrier is semi-permeable to allow entry of peripheral peptides, such as insulin and leptin, into the CNS. Neurons within the ARC are therefore directly exposed to peripheral hormones **(Levin et al. 2011)**. ARC neurons are connected to other hypothalamic areas, such as the PVN, the DMH, and the LHA. These hypothalamic areas further integrate the signals received from the ARC in order to regulate feeding behavior and energy expenditure.



PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; VMN, ventromedial nucleus; LH, lateral hypothalamus; ARC, arcuate nucleus; VTA, ventral tegmental area; DRN, dorsal raphe nucleus; NTS, nucleus of the solitary tract. Projections between nuclei are indicated with arrows. Sites of action of neuropeptides and neurotransmitters inhibiting (−) or stimulating (+) food intake are shown. CART, cocaine- and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; GLP-1, glucagon-like peptide 1; MCH, melanin-concentrating hormone; OXT, oxytocin; AVP, vasopressin; TRH, thyrotropin-releasing hormone; NA, noradrenaline; A, adrenaline; PrRP, prolactin-releasing peptide; $PYY_{3–36}$, peptide YY residues 3–36; OXM, oxyntomodulin; CCK, cholecystokinin. MC3R and MC4R, melanocortin receptors; NPY1R and NPY5R, NPY receptors; LEPRb, long 'signaling' isoform of the leptin receptor; INSR, insulin receptor; GHSR, ghrelin receptor; GLP-1R, GLP-1 receptor. 3V, third ventricle.

Figure 4. Schematic representation of the hypothalamic nuclei and other higher brain regions **(Yeo and Heisler 2012)**. See text for details.

The role of the ARC in feeding and energy homeostasis

The ARC contains two distinct neuronal populations, which either stimulate appetite or cause cessation of feeding. Appetite stimulatory neurons express orexigenic neuropeptides, such as neuropeptide Y (NPY) and Agouti-related peptide (Agrp), while appetite inhibitory neurons express the anorexigenic neuropeptide pro-opiomelanocortin (POMC) **(Cone et al. 2001, Neary et al. 2004, Arora and Anubhuti 2006)**. Receptors for NPY are G-protein coupled receptors, which are termed Y1, Y2, Y4, Y5, and Y6 **(Wahlestedt and Reis 1993, Gehlert 2004)**. Agrp on the other hand is a potent antagonist for melanocortin 3 and 4 receptors (MC-3R and MC-4R) **(Yang 2014)**. POMC is cleaved by endopeptidases to generate α-melanocyte stimulating

hormone (α-MSH), which is an agonist for MC-3R and MC-4R **(Pritchard et al. 2002)**. Neurons within the PVN, DMH, and LHA express NPY receptors, MC-3R and MC-4R and their activity is therefore directly modulated by Agrp, NPY and POMC to regulate feeding behavior **(Xia and Wikberg 1997, Fetissov et al. 2004, Siljee et al. 2013) (Figure 4)**.

The most important peripheral hormones that regulate feeding behavior and energy homeostasis are leptin, insulin, and ghrelin. Leptin is secreted from adipose tissue and its circulating levels are proportional to adipose tissue mass **(Considine et al. 1996)**. Insulin is secreted from pancreatic β-cells in response to increased post-prandial blood glucose levels **(Komatsu et al. 2013)**. Importantly, Agrp/NPY and POMC neurons express the insulin receptor (IR) and the leptin receptor (LR), which enables these neurons to respond to these peripheral hormones **(Havrankova et al. 1978, Meister 2000)**. Since insulin and leptin levels rise under nutrient rich conditions, both hormones induce an appetite-inhibitory, i.e. anorexigenic, response in the ARC. Leptin and insulin stimulate anorexigenic POMC neurons and inhibit orexigenic Agrp/NPY neurons, resulting in increased energy expenditure and cessation of feeding **(Varela and Horvath 2012)**. On a molecular level, leptin and insulin stimulate *POMC* and inhibit *Agrp* and *NPY* expression via the signal transducer and activator of transcription 3 (STAT3) and the PI3K/Akt/ FOXO1 signaling pathways **(Figure 5)**. When leptin binds to its receptor, Janus kinases (JAKs) are recruited to the leptin receptor, leading to its phosphorylation and activation. This allows binding of STAT3 to the LR. JAKs subsequently phosphorylate STAT3, which results in STAT3 dimerization and translocation to the nucleus. In the nucleus, the STAT3 dimer stimulates *POMC* transcription in POMC neurons and inhibits *Agrp* and *NPY* transcription in Agrp/NPY neurons **(Mesaros et al. 2008, Ernst et al. 2009)**. Insulin binds to the IR and activates PI3K/Akt signaling. Akt is a negative regulator for FOXO1, causing its nuclear exclusion upon its phosphorylation **(Matsuzaki et al. 2003)**. In

Agrp/NPY neurons, FOXO1 stimulates *Agrp* and *NPY* expression, while it inhibits *POMC* expression in POMC neurons **(Kitamura et al. 2006, Plum et al. 2009)**. Hence, upon insulin stimulation FOXO1 is phosphorylated and therefore excluded from the nucleus, which results in a decrease in *Agrp* and *NPY* expression and an increase in *POMC* expression.

In contrast to these anorexigenic peptides, ghrelin induces an orexigenic response. Ghrelin is mainly synthesized in endocrine cells of the gastric mucosa and it acts via stimulation of Agrp and NPY production. Ghrelin also antagonizes the inhibitory effect of leptin on Agrp/NPY neurons **(Greenman et al. 2004)**. Hence, ghrelin causes an induction of feeding and an increase in energy expenditure. However, the molecular signaling pathways mediating the effects of ghrelin on *Agrp* and *NPY* expression are still poorly understood.

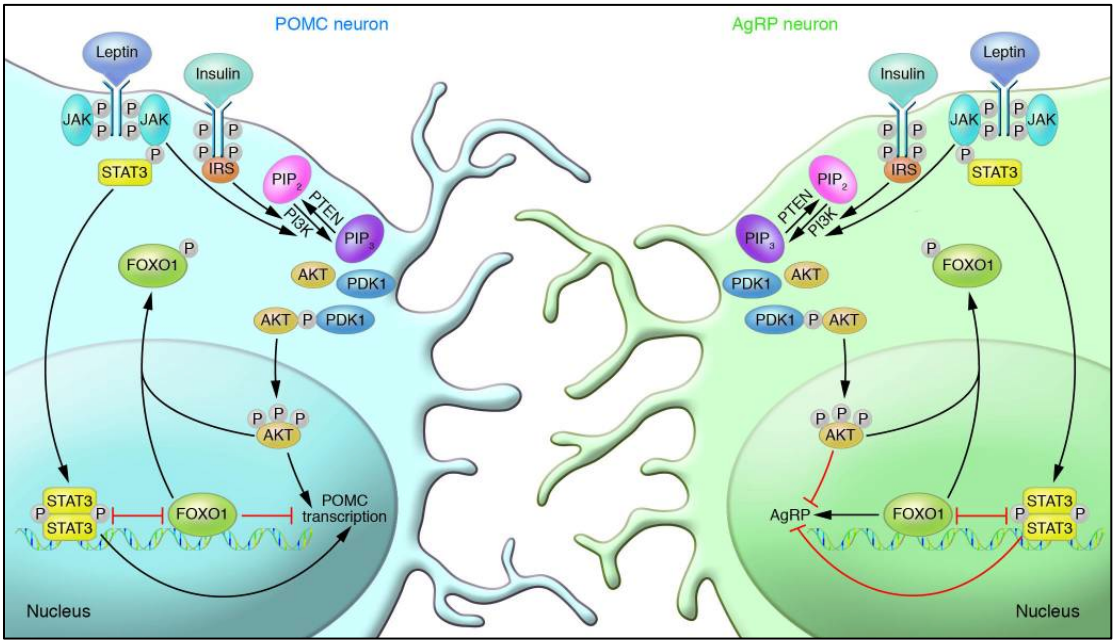


Figure 5. Insulin and leptin regulate Agrp and POMC expression **(Plum et al. 2006)**. See text for details.

A potential role for mTOR signaling in the hypothalamus

As mentioned earlier, mTOR is a nutrient and energy sensor and therefore a crucial regulator of metabolism and energy homeostasis. In particular, insulin is an important growth factor which regulates both mTORC1 and mTORC2 activity. Since, *Agrp/NPY* and *POMC* expression is also regulated by insulin it has been hypothesized that

mTOR signaling plays an important role in the hypothalamic regulation of feeding behavior and energy homeostasis. Indeed, mTORC1 activity in the ARC is low in fasted animals and high during feeding, suggesting that hypothalamic mTORC1 activity is sensitive to the feeding state **(Cota et al. 2006)**. Moreover, mTORC1 activity is increased specifically in POMC but not Agrp/NPY neurons in aged mice, leading to POMC neuronal dysfunction and thereby contributing to aging-induced obesity **(Yang et al. 2006)**. Importantly, POMC-specific *TSC1* KO (POMC-*TSC1* KO) mice with chronic mTORC1 hyper-activation in POMC neurons, display hyperphagia-induced obesity due to decreased *POMC* expression **(Mori et al. 2009) (Table 1)**. Interestingly, POMC-specific rictor KO (POMC-*rictor* KO) mice, which display inactive mTORC2 signaling in POMC neurons, also develop an obese and hyperphagic phenotype **(Kocalis et al. 2014) (Table 1)**. Thus, the hyperphagic phenotype of POMC-*rictor* KO and POMC-*TSC1* KO mice could be due to impaired Akt signaling. As mentioned in the previous section, Akt is a negative regulator of FOXO1. In POMC-*rictor* KO and POMC-*TSC1* KO mice Akt signaling is impaired, which leads to nuclear localization and activation of FOXO1. Activated FOXO1 represses *POMC* expression, resulting in decreased POMC levels and hyperphagia. Surprisingly, inactivation of mTORC2 specifically in Agrp neurons does not result in any alterations in feeding behavior or energy homeostasis **(Kocalis et al. 2014)**. Hence, mTORC2 signaling is a crucial regulator of POMC neuronal function, which affects both feeding behavior and systemic energy homeostasis, while it is dispensable for Agrp neuronal function.

mTOR in adipose tissue

Adipose tissue is a heterogeneous organ consisting of adipocytes, preadipocytes, fibroblasts, endothelial cells, and immune cells, such as macrophages, B- and T-cells. There are three different types of adipose tissue: white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue, also termed “brown-in-white” (brite) adipose tissue **(Figure 6)**. White, brown and beige adipocytes arise

from different progenitors and they have distinct functions. However, all types of adipose tissue have a crucial role in the regulation of whole-body energy homeostasis.

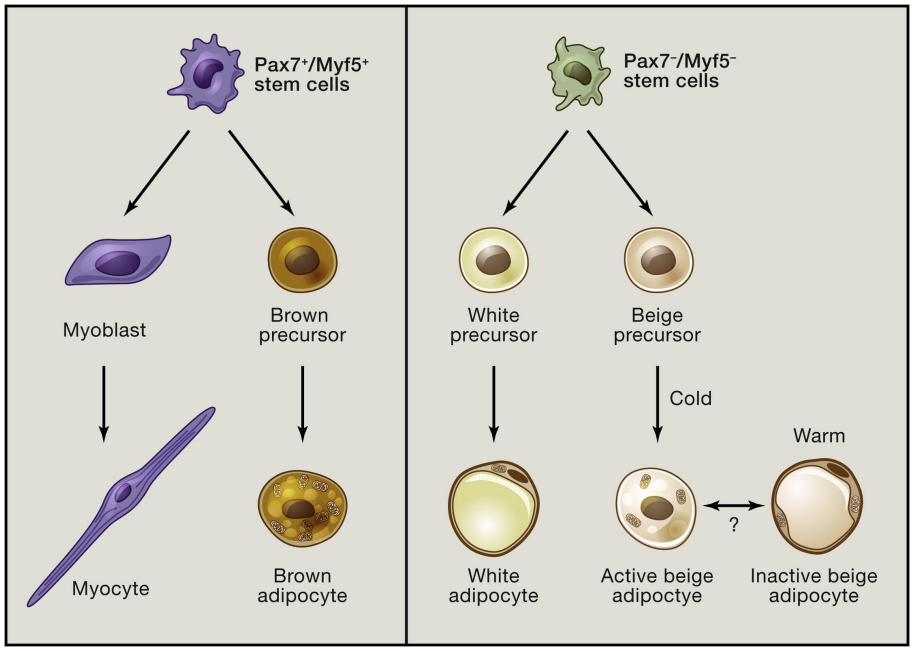


Figure 6. Origin of brown, white and beige adipocytes **(Rosen and Spiegelman 2014)**. See text for details.

Origin and function of WAT

White adipocytes arise from preadipocytes, which originate from mesenchymal stem cells that have undergone an early commitment to the adipocyte lineage **(Rosen and MacDougald 2006) (Figure 6)**, a process that is not yet fully elucidated. Subsequently, upon terminal differentiation, white preadipocytes acquire the functional properties of mature white adipocytes. This terminal differentiation process is tightly regulated by the expression of key transcription factors involved in adipogenesis, such as CCAAT/enhancer binding protein α, β, and δ (C/EBPα, β, and δ) and peroxisome proliferator-activated receptor γ (PPARγ) **(Rosen and MacDougald 2006)**.

Energy storage and release. The main function of WAT is to store energy and excess nutrients in the form of triglycerides (TGs) when nutrient availability is high. White adipocytes are characterized by a single large lipid droplet where TGs are stored, which can account for over 90% of the cell volume **(Haugen and Drevon 2007) (Figure**

6). TGs that are stored in WAT are mainly derived from dietary fats, and only a small fraction is generated by *de novo* lipogenesis in adipocytes. Dietary fat is digested in the stomach and small intestine to yield fatty acids (FAs). In enterocytes of the intestine FAs are re-synthesized to TGs, packaged into chylomicrons, and released into the circulation. Chylomicrons are lipoproteins containing TGs, cholesterol and vitamins, specialized in transporting lipids in the circulation. FAs are released from chylomicrons by lipoprotein lipases that are expressed in endothelial cells of the capillaries within WAT depots. The released FAs then diffuse into adipocytes where they are re-synthetized to TGs by esterification with glycerol 3-phosphate and stored within lipid droplets. Additionally, excess carbohydrates can also be converted to TGs via *de novo* lipogenesis in both liver and WAT and stored within lipid droplets **(Penicaud et al. 2000)**.

Stored TGs can be mobilized from lipid droplets by lipases, such as hormone sensitive lipase (HSL), adipose tissue TG lipase (ATGL) and monoglyceride lipase (MAG) in a process termed lipolysis **(Duncan et al. 2007)**. Lipolysis is induced in WAT upon β-adrenergic stimulation, and involves activation protein kinase A (PKA), which phosphorylates and stimulates HSL activity. The FAs generated through lipolysis are released into the blood stream and transported to other organs. Hence, lipolysis in WAT is crucial to provide energy for other organs in times of low nutrient availability, such as during starvation or when systemic energy demand is increased, for example during intense exercise or upon cold exposure.

Endocrine function. WAT exerts many of its effects on systemic energy homeostasis and metabolism via secretion of a variety of adipose-derived hormones, termed adipokines, which act on other organs **(Trayhurn and Beattie 2001, Trayhurn and Wood 2004)**. Adipokines include growth- and angiogenic factors, chemokines, cytokines, adhesion molecules and hormones **(Figure 7)**. Through these adipokines, adipose tissue is able to affect a variety of systemic processes ranging from angiogenesis, immune response, glucose

homeostasis and feeding behavior **(Trayhurn and Wood 2004)**. Importantly, deregulation of WAT function, for example in an obese or diabetic state, leads to aberrant adipokine secretion, thereby having a major impact on systemic metabolic homeostasis.

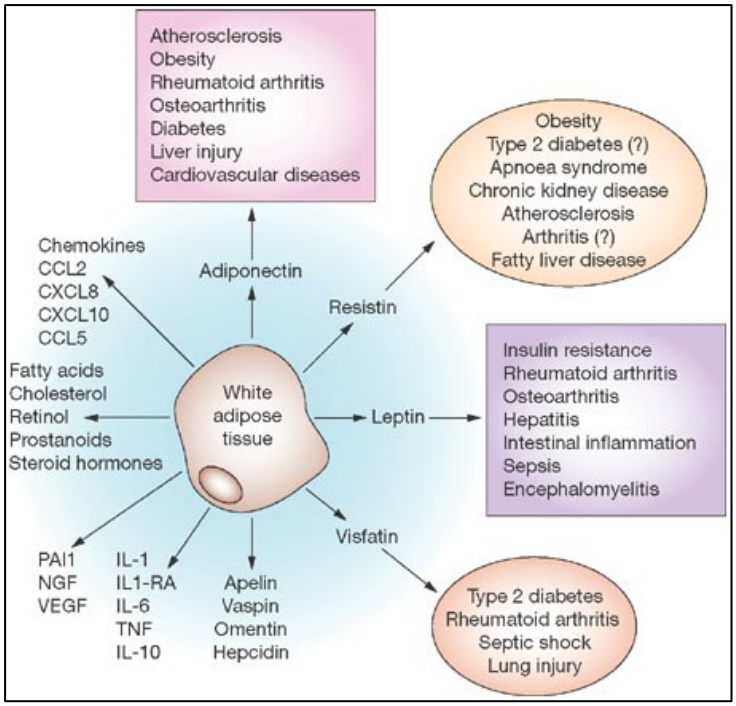


Figure 7. Adipokines secreted by WAT **(Lago et al. 2007)**.

Regulation of inflammation. Recently, it has become evident that WAT plays an important role in immune function, thereby contributing to the regulation of both local and systemic inflammation **(Rosen and Spiegelman 2014)**. This has become apparent since WAT actively secretes cytokines, such as tumor necrosis factor α (TNF-α) or interleukin 6 (IL-6) **(Hotamisligil et al. 1993)**. Under obese conditions, the secretion of these pro-inflammatory cytokines is enhanced, which contributes to diet-induced adipose tissue and systemic insulin resistance **(Hotamisligil et al. 1993)**. Interestingly, more than 60% of all cells within a WAT depot can be of immune origin **(Kanneganti and Dixit 2012)**, suggesting that the majority of cytokines secreted from WAT are secreted by immune cells and not by adipocytes **(Weisberg et al. 2003, Xu et al. 2003)**. Macrophages play a particularly important role in WAT immune function. There are two different types of macrophages, which are termed M1 (classically

activated) and M2 (alternatively activated) **(Mills 2012)**. M2 macrophages display an anti-inflammatory phenotype and they are the dominating macrophage-population in WAT under lean conditions. Under obese conditions, the number of pro-inflammatory M1 macrophages strongly increases, resulting in an elevated secretion of pro-inflammatory cytokines and impaired insulin sensitivity **(Lumeng et al. 2007, Lumeng et al. 2007)**. In line with this, overexpression of monocyte-chemoattractant protein 1 (MCP-1) leads to macrophage-infiltration and insulin resistance while its inhibition ameliorates insulin resistance in obese mice **(Kamei et al. 2006, Kanda et al. 2006)**. In contrast to this, TNF-α KO mice that lack an immune response, display a worsened insulin resistance upon diet-induced obesity (DIO) **(Wernstedt Asterholm et al. 2014)**. Hence, the precise role of adipose tissue inflammation in obesity is not yet completely understood. However, the above-mentioned observation suggests that adipose tissue inflammation is not necessarily only detrimental and can also exert positive effects on metabolic health in obese conditions.

Origin and function of BAT

BAT develops during embryogenesis prior to the formation of other adipose tissue depots. Most brown adipocytes arise from myogenic factor 5 (Myf5) and paired box protein 7 (Pax7) double-positive progenitor cells, which also give rise to skeletal muscle cells **(Seale et al. 2008) (Figure 6)**. Hence, brown adipocytes are more closely related to skeletal muscle myocytes than to white adipocytes. Commitment of Myf5 positive progenitors to the brown adipocyte lineage is mediated by PRD1-BF-1-RIZ1 homologous domain-containing protein-16 (PRDM16) **(Seale et al. 2007, Seale et al. 2008)**. PRDM16 directly binds to the DNA and induces expression of BAT-characteristic genes, such as PPARγ coactivator 1α (PGC-1α), uncoupling protein 1 (UCP1), and type II iodothyronine deiodinase (Dio2). Importantly, PRDM16 also inhibits expression of several white adipocyte specific genes, such as resistin, angiotensinogen, endothelin

receptor type A (Ednra) and phosphoserine aminotransferase (Psat) **(Kajimura et al. 2008)**.

Non-shivering thermogenesis (NST) and energy dissipation. In contrast to the energy storing properties of WAT, BAT is mainly involved in temperature homeostasis and NST by dissipating energy in the form of heat. NST allows mammals to maintain stable body temperature in a cold environment. Upon cold exposure, norepinephrine (NE) is released from sympathetic nerves and binds to β_3-adrenergic receptors on brown adipocytes to induced NST. β_3-adrenergic receptor stimulation induces cAMP production and subsequent activation of PKA. PKA signaling then stimulates lipid mobilization, β-oxidation and mitochondrial uncoupling **(Cannon and Nedergaard 2004) (Figure 8)**.

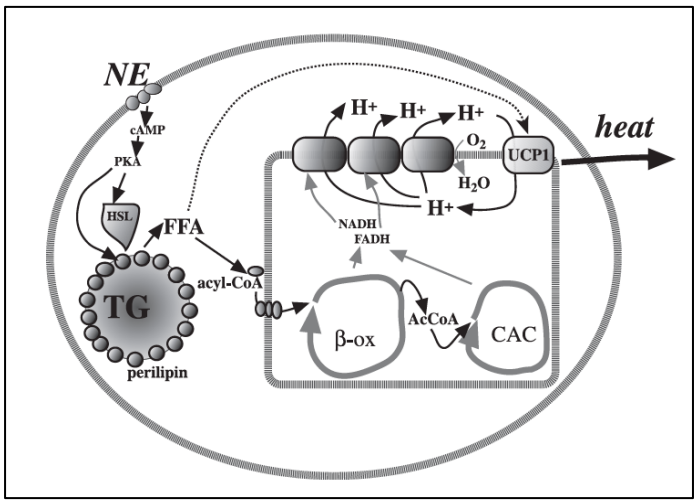


Figure 8. β-adrenergic stimulation induces non-shivering thermogenesis in BAT **(Cannon and Nedergaard 2004)**. See text for details.

Thus, brown adipocytes are characterized by possessing a large number of mitochondria and many small lipid droplets that can be easily mobilized to generate substrates for mitochondrial uncoupling **(Figure 6)**. Mitochondrial uncoupling is achieved by activating UCP1, which is a member of the mitochondrial carrier protein family and specifically expressed in brown adipocytes. It is a transmembrane protein found in the mitochondria and its function is to dissipate the proton gradient across the inner mitochondrial membrane generated by the electron transport chain (ETC). This uncouples the proton flux into

the mitochondria from ATP production, resulting in heat generation **(Klaus et al. 1991, Busiello et al. 2015)**. When NST is not active, UCP1 is inhibited by cytosolic nucleotides, such as GDP, GTP, ADP and ATP **(Nicholls 1974)**. However, upon β-adrenergic stimulation, UCP1 activity is stimulated by FAs via a still not fully understood mechanism **(Rial et al. 1983)**. Either FAs act as direct cofactors for UCP1 or they increase UCP1 activity by preventing binding of inhibitory cytosolic nucleotides. Consequently, release of FAs is essential for NST and defects in FA uptake, FA transport or lipolysis result in impaired UCP1 activity and reduced heat generation upon cold stress **(Haemmerle et al. 2006, Wu et al. 2006, Vergnes et al. 2011)**. Additionally, β-adrenergic stimulation induces UCP1 transcription upon cold stress, resulting in increased UCP1 protein levels and thereby an enhanced thermogenic capacity. Due to the ability of BAT to efficiently burn energy, activation of NST and subsequent energy dissipation has been proposed as a strategy to induce weight loss in obese patients **(Clapham and Arch 2011)**. Hence, a large amount of work has gone into identifying novel regulators of NST. Table 2 summarizes the most important factors in BAT that can affect thermogenesis. Importantly, most of the identified proteins modulate expression of thermogenic genes, such as UCP1, thereby regulating energy dissipation.

Gene	Induces beige fat	Increases brown fat	Comments
Gain-of-function models			
Ptgs2 (also known as *Cox2*)	Yes	Not determined	Cox2-overexpressing mice have increased beige fat and are resistant to weight gain, demonstrating the role of prostaglandins in the recruitment of beige fat.
Foxc2	Yes	Yes	Overexpression of Foxc2 in adipose increases the expression of the R1α regulatory subunit of PKA, making the cells more sensitive to catecholamines.
Prdm16	Yes	No	Mice selectively transgenic for Prdm16 in fat have increased beige fat.
Pten	Yes	Yes	Increases in Pten levels inhibit PI3K, which drives a thermogenic program.
Ucp1	Yes	No	Transgenic expression of *Ucp1* increases thermogenesis in WAT and prevents weight gain.
Loss-of-function models			
Acvr2b	No	Yes	Neutralizing antibodies to ActRIIB lead to an increase in BAT mass without affecting WAT. Loss of ActRIIB activates Smad3 signaling to increase expression of thermogenic genes.
Adrbk1	Yes	Yes	Increased core temperature and thermogenic program in BAT and WAT. Interestingly, the phenotype seems to be age related.
Acot11 (also known as *Them1*)	No	Yes	Increased expression of in thermogenic genes in BAT and a decreased expression of markers of inflammation in WAT.
Aldh1a1	Yes	No	Build up of retinaldehyde leads to activation the retinoic acid receptor, which recruits Pgc-1α to the *Ucp1* promoter.
Arrdc3	Yes	Yes	Arrdc3 interacts directly with β-ARs. Loss of Arrdc3 sensitized adipocytes to catecholamines and thus increased thermogenic programs in BAT and WAT.
Atg7	Yes	Yes	BAT showed increased amounts of thermogenic proteins, and WAT had increased expression of thermogenic gene signatures. Studies have demonstrated a role for autophagy in adipose development.
Atf4	Yes	Yes	WAT showed increased expression of Pgc-1α and Ucp2, and BAT was enriched for expression of Ucp1 and Ucp3.
Bace1	No	Yes	Increased expression of Ucp1 in BAT and of Ucp2 and Ucp3 in skeletal muscle.
Cidea	No	Yes	Knockout (KO) mice are lean, have increased oxygen consumption and defend core temperature against cold challenge. Direct interactions with Ucp1 could explain the repressive effect of Cidea.

Table 2. Mouse models with increased NST **(Harms and Seale 2013)**.

Gene	Induces beige fat	Increases brown fat	Comments
Cidec	Yes	No	Increased expression of BAT-specific genes and of mitochondrial genes in WAT. The mechanism is thought to involve loss of pRb and Rip140.
Cnr1	Yes	Not determined	KO mice are lean. *In vitro*, cannabinoid receptor type 1m antagonists can induce *Ucp1* transcription in white adipocytes.
Crfr2 (also known as *Crhr2*)	Not determined	Yes	Increased glucose tolerance and increased Ucp1 expression in BAT.
Dlk1 (also known as *Pref1*)	Not determined	Yes	BAT has increased expression of Pgc-1α and Ucp1. C/ebpβ binds and activates the *Pref1* promoter.
Eif4ebp1	Yes	No	Increased metabolic rate, induction of thermogenic genes in WAT depots and increased eIF4F phosphorylation.
Eif4ebp2	No	Yes	Treatment with an antisense oligonucleotide to *Eif4ebp2* caused weight loss and increased expression of the β3-AR in WAT and BAT. BAT showed a Pgc-1α–independent increase in Ucp1 expression.
Ffar2	Not determined	Yes	Resistance to weight gain and increased core temperature.
Foxo1	Not determined	Yes	Mice expressing a dominant-negative form of Foxo1 in their adipose tissue had increased oxygen consumption and a BAT-specific increase in thermogenesis.
Ghsr	Not determined	Yes	Mice are protected from the age-associated decline of thermogenesis.
Id1	No	Yes	Increased oxygen consumption and an increased expression of thermogenic genes in BAT.
Ikbke	Yes	Not determined	WAT has increased amounts of Ucp1 transcripts and protein.
Lipe	Yes	Not determined	The increased expression in Ucp1 is attributed to a decrease in Rip140 and pRb expression.
Lrp6	Yes	Yes	KO mice gain less weight than controls, and diminished mTORC1 activity in BAT causes an increase in the expression of thermogenic proteins.
Mstn	Yes	Not determined	Increased thermogenic program in WAT.
Npr3	Yes	Yes	Loss of the natriuretic peptide clearance receptor causes increased concentrations of circulating natriuretic peptides, which increase thermogenic activity.
Ncoa2 (also known as *Tif2*)	Not determined	Yes	Tif2 competes with the activator Src2 for Pgc-1α binding. Tif2 binding prevents Pgc-1α from interacting with Ppar-γ.
Nr1h3 (also known as *Lxra*)	Yes	Yes	RIP140 is recruited by Lxra to displace Ppar-γ. Pgc-1α also binds to this site, but its occupancy doesn't seem to change.
Nrip1 (also known as *Rip140*)	Yes	No	Rip140 interacts directly with Pgc-1α to inhibit its transcriptional activity. Rip140 recruits chromatin modifiers such as DNA methyltransferases and histone methyltransferases to silence *Ucp1*.
Oprd1	Not determined	Yes	Mice are resistant to weight gain and have enhanced thermogenesis in BAT.
Pctp	Not determined	Yes	BAT showed enlarged mitochondria and an increased expression of thermogenic genes.
Prkar2b	Not determined	Yes	Loss of Prkar2b causes a compensatory increase in the amount of RIα, which binds cAMP with higher affinity than Pkar2b, causing increased basal PKA activity and increased thermogenesis.
Prkcb	Yes	Yes	WAT had increased expression of β1 and β3 adrenergic receptors. This resulted in a p38-MAPK–mediated increase in the expression of *Pgc1a* and *Ucp1*.
Prlr	Yes	Not determined	Prolactin-receptor KO mice have increased expression of thermogenic genes and altered amounts of pRb and Foxc2 in WAT. This indicates a new paracrine or endocrine role of prolactin.
Rbl1 (also known as *p107*)	Yes	Yes	Loss of p107 causes a loss of pRb and increased expression of thermogenic genes.
Scd1	Yes	Yes	Mice with skin-specific KO of *Scd1* have increased thermogenesis in BAT and WAT, indicating crosstalk between the different tissues
Sfrp5	Yes	Not determined	KO mice are resistant to weight gain, and isolated KO adipocytes have increased oxidative respiration.
Smad3	Yes	No	Smad3 represses Pgc-1α expression. Loss of Smad3 induces the expression of transcripts that correspond to increased thermogenesis.
Tnfrsf1a	Not determined	Yes	Increased expression of Ucp1 in BAT and of Ucp3 in muscle, resulting in increased oxygen consumption.
Trpv4	Yes	Not determined	KO mice are resistant to weight gain and have increased thermogenic gene expression in WAT mediated by a loss of ERK1- and ERK2-mediated effects on Pgc-1α.
Twist1	Not determined	Yes	Twist 1 binds to and inhibits Pgc-1α activity at target genes.
Vegfa	Yes	No	Induction of the thermogenic program in WAT with associated resistance to weight gain.
Vgf	Yes	Yes	KO of the secreted protein Vgf caused increased expression of Ucp1 in WAT and BAT. Unclear whether the effect is cell autonomous.

Table 2 (continued)**.** Mouse models with increased NST **(Harms and Seale 2013)**.

Glucose homeostasis. Besides the effects on lipid metabolism, activation of NST also has major impact on systemic glucose homeostasis. During NST, ATP is generated in BAT through glycolysis to compensate for the loss of mitochondrial ATP production. Hence, glucose uptake into BAT is strongly enhanced upon cold exposure **(Greco-Perotto et al. 1987, Vallerand et al. 1990)**. Interestingly, cold-induced glucose uptake into BAT is induced by β-adrenergic stimulation via an insulin-independent mechanism **(Shibata et al. 1989)**. It has therefore been suggested that activation of NST could be used to normalize blood glucose levels in diabetic, insulin-resistant patients. However, in contrast to insulin-induced glucose uptake, which is mediated through Akt-stimulated translocation of GLUT4, much less is known about the mechanism of β-adrenergic stimulation-induced glucose uptake. β-adrenergic stimulation-induced glucose uptake is mediated by members of the GLUT transporter family **(Santalucia et al. 1992)**. However, it is not clear whether GLUT1 or GLUT4 or both mediate NST-induced glucose uptake. Additionally, it has not been fully elucidated whether β-adrenergic stimulation-induced glucose uptake involves translocation of glucose transporters to the plasma membrane or whether the transporters constantly reside in the plasma membrane where they become activated upon β-adrenergic stimulation, for example through post-translational modifications (PTMs) **(Palmada et al. 2006)**. Hence, in order to use β-adrenergic stimulation-induced glucose uptake as a treatment option for diabetic patients, it is of importance to elucidate the molecular signaling pathways mediating this process.

Origin and function of beige adipose tissue

Beige adipocytes are the most recently identified adipocyte type. They mainly reside within subcutaneous WAT (sWAT) depots and display an intermediate phenotype between white and brown adipocytes **(Figure 6)**. The origin of beige adipocytes is still not fully understood. Beige adipocytes are clearly distinct from brown adipocytes, because they do

not originate from Myf5 positive precursor cells **(Seale et al. 2008)**. Moreover, beige adipocytes express a unique gene signature. For example, the surface markers CD137 and transmembrane protein 26 (TMEM26) are specifically expressed in beige but not in brown or white adipocytes **(Wu et al. 2012)**. However, it is still unclear whether beige adipocytes are a distinct cell type or whether they arise via transdifferentiation of white adipocytes **(Rosen and Spiegelman 2014)**.

Beige adipocytes were discovered due to the observation that prolonged cold exposure resulted in the appearance of UCP1-positive cells with brown adipocyte-like morphology within WAT depots. In the absence of thermogenic stimuli, beige adipocytes serve as energy stores and they display similar rates of uncoupling and energy expenditure as white adipocytes. However, under cold stress, when the thermogenic demand of an organism is increased, beige adipocytes undergo browning. Upon browning, β-adrenergic stimulation induces expression of brown adipocyte-characteristic genes, such as *PGC-1α*, *UCP1*, and *Dio2* in beige adipocytes **(Loncar et al. 1988, Loncar 1991, van Marken Lichtenbelt et al. 2009, Frontini and Cinti 2010, Rosenwald et al. 2013)**. Consequently, browning leads to heat production in beige adipocytes and is therefore a crucial process to enhance the thermogenic capacity of an organism under chronic cold exposure.

mTOR affects adipose tissue biology

mTORC1 signaling in adipose tissue plays an important role in regulating whole body energy expenditure, systemic insulin sensitivity, and thermogenesis **(Table 1)**. Adipose tissue-specific raptor KO (AdRaKO) mice with impaired mTORC1 signaling in adipose tissue are lean and protected from diet-induced obesity due to increased uncoupling in WAT **(Polak et al. 2008)**. Moreover, AdRaKO mice display enhanced systemic insulin sensitivity. Conversely, adipose tissue-specific *Grb10* KO mice, which display increased mTORC1

signaling in adipose tissue, have decreased energy expenditure due to impaired uncoupling and thermogenesis in BAT and WAT, which leads to obesity and insulin resistance **(Liu et al. 2014) (Table 1)**. Thus, hyper-activation of mTORC1 signaling in adipose tissue exerts a negative effect on whole body metabolism and systemic energy homeostasis.

mTORC2 signaling in adipose tissue also affects whole body metabolism by influencing systemic insulin sensitivity, systemic glucose homeostasis and whole-body growth **(Table 1)**. Adipose-tissue specific *rictor* KO (AdRiKO) mice, which display impaired mTORC2 signaling in adipose tissue, have a strong increase in circulating insulin due to an enlarged pancreas **(Cybulski et al. 2009)**. This substantial increase in circulating insulin results in improved systemic glucose tolerance despite slight insulin resistance in skeletal muscle and liver. Moreover, upon HFD feeding, AdRiKO mice display an increase in lean mass, which results in increased body size **(Cybulski et al. 2009)**. Inactivation of mTORC2 signaling specifically in Myf5 positive muscle/brown adipocyte progenitors on the other hand increases oxidative metabolism in BAT and protects the mice from diet-induced obesity due to increased uncoupling without affecting body size **(Hung et al. 2014) (Table 1)**.

1.3 mTOR in disease

Due to its central role in growth and metabolism, deregulation of mTOR signaling is involved in the development of several diseases **(Dazert and Hall 2011, Laplante and Sabatini 2012)**. Figure 9 shows an overview of diseases that have been associated with deregulated mTOR signaling. Importantly, many of these diseases are age-related, suggesting that mTOR signaling affects aging. Indeed, pharmacological or genetic inhibition of TORC1 signaling results in an increased life span in yeast, worms, flies and an increased life- and health span in mammals **(Vellai et al. 2003, Kapahi et al. 2004, Kaeberlein et al. 2005, Powers et al. 2006, Chen et al. 2009, Harrison et al. 2009, Anisimov et al. 2010, Bjedov et al. 2010, Miller**

et al. 2011, Robida-Stubbs et al. 2012, Wilkinson et al. 2012). Thus, mTOR is an attractive target for the treatment of age-related diseases and to slow aging.

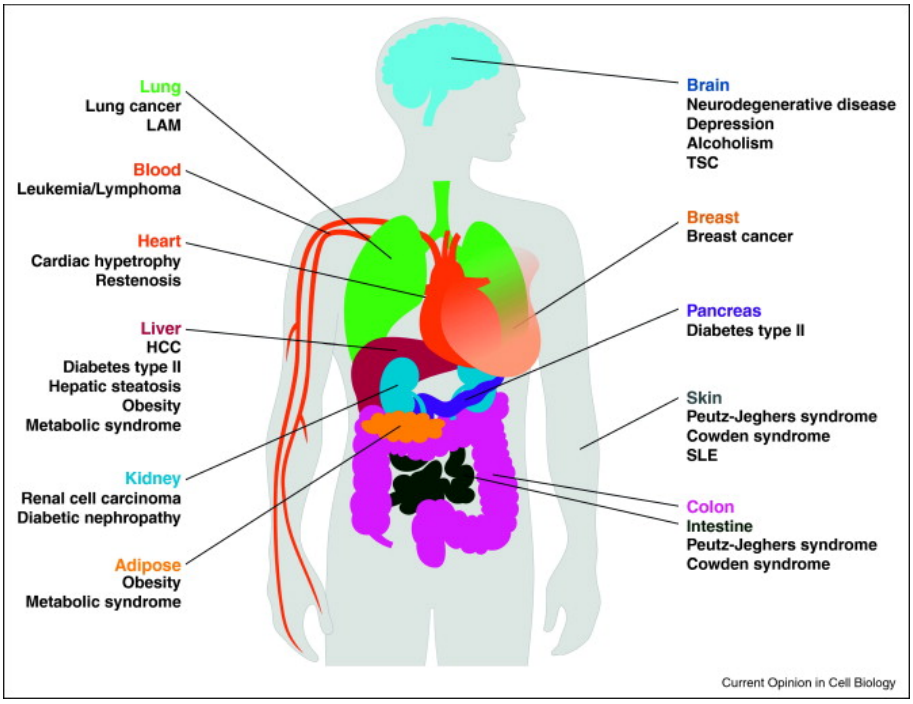


Figure 9. Diseases linked to deregulated mTOR signaling **(Dazert and Hall 2011)**.

mTOR in cancer

Deregulation of mTOR signaling plays a crucial role in the development of cancer. Due to the importance of mTOR in regulating cell growth, hyper-activation of mTOR signaling promotes proliferation and cell survival and leads therefore to tumor development. It has been estimated that a high percentage of all cancers displays hyper-activation of mTOR signaling **(Shaw and Cantley 2006, Roberts and Der 2007)**. Interestingly, while mTOR itself is rarely mutated, upstream negative regulators of mTOR signaling, such as TSC1/2 and phosphatase and tensin homolog (PTEN) are frequently inactivated in human tumors **(Simpson and Parsons 2001, Curatolo et al. 2008)**. In line with this, genetically modified mice with TSC1/2 or PTEN deletion display hyperactive mTOR signaling and consequently develop tumors **(Yeung et al. 1997, Di Cristofano et al. 1998, Suzuki et al. 1998, Podsypanina et al. 1999, Backman et al. 2001, Kwiatkowski et al. 2002, Trotman et al. 2003, Wang et al. 2003,**

Horie et al. 2004, Wenzel et al. 2004, Meikle et al. 2005, Freeman et al. 2006, Meikle et al. 2007, Guertin et al. 2009, Menon et al. 2012, Kenerson et al. 2013). These studies demonstrate that hyper-activation of mTOR signaling is a strong driver of tumorigenesis.

Many downstream processes regulated by mTOR signaling, such as translation, *de novo* lipogenesis, and cell survival, have been shown to be involved in cancer development **(Laplante and Sabatini 2012)**. For example, the mTORC1-4E-BP1-eIF4E signaling pathway specifically promotes translation of pro-oncogenic mRNAs involved in proliferation and metastasis **(Thoreen et al. 2012)**. Moreover, proliferating cancer cells are characterized by an increase in *de novo* lipogenesis in order to generate lipids that are required for the synthesis of membranes **(Menendez and Lupu 2007)**. As mentioned earlier, both mTORC1 and mTORC2 stimulate lipogenesis through regulation of SREBPs **(Duvel et al. 2010, Peterson et al. 2011, Hagiwara et al. 2012, Yuan et al. 2012)**. Interestingly, depletion of SREBPs in *TSC2*-deficient cells blocks cell proliferation, indicating that the lipogenic gene program is required for growth of cells with hyperactive mTORC1 signaling **(Duvel et al. 2010)**. Finally, mTORC2 is a crucial regulator of cell survival through its main downstream target Akt **(Zhang et al. 2011)**.

Due to the central role of mTOR signaling in cancer development, progression and metastasis, the mTOR pathway has become an attractive target for cancer therapy. Thus, many rapamycin analogs and mTOR inhibitors are currently tested in clinical trials against a large variety of cancers **(Wander et al. 2011)**.

mTOR in obesity and diabetes

Metabolic disorders develop due to disturbance in energy homeostasis. In particular, obesity develops when caloric intake exceeds the amount of calories that are burned. Since mTOR signaling is a nutrient sensor and since its activity is modulated by energy, nutrients and growth factors, it has been suggested that deregulation of mTOR signaling plays a major role in the development of metabolic disorders. In line

with this notion, mice with alterations in mTOR signaling in metabolic organs, such as liver, muscle, adipose tissue and hypothalamus, often display an aberrant regulation of metabolism and energy homeostasis (**Table 1**, see above for more details). For example, hyper-activation of mTORC1 or inactivation of mTORC2 in the liver leads to glucose intolerance and insulin resistance **(Sengupta et al. 2010, Kenerson et al. 2011, Yecies et al. 2011, Hagiwara et al. 2012)**. Hyper-activation of mTORC1 or inactivation of mTORC2 in POMC neurons results in hyperphagia-induced obesity **(Mori et al. 2009, Kocalis et al. 2014)**. Additionally, mice with hyperactive mTORC1 signaling in adipose tissue are obese and suffer from metabolic complications **(Liu et al. 2014)**. Interestingly, mice fed a HFD display increased mTORC1 signaling in adipose tissue when compared to mice fed a regular diet **(Polak et al. 2008)**. Collectively, these data suggest that, chronic activation of mTORC1 signaling in adipose tissue, liver or hypothalamus could contribute to the development of diet-induced metabolic dysfunction.

To understand the molecular mechanism(s) involved in the development of metabolic disorders, such as cancer, obesity and diabetes, it is important to study the role of mTOR signaling in different metabolic organs. Our knowledge on mTOR signaling in regulating cell growth and metabolism has dramatically increased in the past couple of years. However, there are still many unanswered questions and growing evidence suggests that mTOR signaling has distinct roles in different organs **(Albert and Hall 2014)**. Thus, unraveling novel mTOR-regulated processes will hopefully provide new insights into the development of metabolic disorders, and help for the design of novel treatments against these diseases.

Chapter 2: Aims of the thesis

2. Aims of the thesis

The overall aim of this thesis was to study mTOR signaling in metabolic organs in order to elucidate the impact of organ-specific mTOR signaling on organismal energetics. To address this question, we focused on mTORC1 in the hypothalamus and on mTORC2 in adipose tissue and studied their role in regulating systemic energy homeostasis.

In the first part of this thesis, we investigated the role of mTORC1 signaling in hypothalamic Agrp neurons with particular focus on the impact of impaired mTORC1 signaling in these neurons on feeding behavior and whole-body energy metabolism. In the second part of this thesis we studied whether and how mTORC2 signaling in adipose tissue regulates thermogenesis and whole body energy expenditure. With these studies we hope to provide a better understanding of the molecular mechanisms involved in the regulation of systemic energy homeostasis. This knowledge could help develop novel treatment strategies against obesity and obesity-associated diseases. Obesity is a strong risk factor for the development of several other diseases, such as diabetes, cardiovascular disease and cancer. Alarmingly, the prevalence of obesity has drastically increased during the last decades in both developing and developed countries **(Ng et al. 2014)**. Since obesity develops due to a sedentary lifestyle and an excess of calorie intake, an efficient and cost-effective way to ameliorate metabolic dysfunction in obese patients would be to increase physical activity and reduce calorie intake in the patients. However, maintaining diet and exercise regimens is challenging and has therefore shown only moderate success as an anti-obesity treatment in obese patients. In light of this, there is a need for novel therapies aimed at reducing obesity, and thereby reducing the prevalence of metabolic diseases in the population.

Recently, it has become evident that dysfunction of hypothalamic neurons involved in feeding behavior often contributes to the development of obesity **(Velloso et al. 2009)**. For example, many obese patients display leptin and insulin resistance in Agrp and POMC neurons, which

deregulates normal feeding behavior and thereby leads to metabolic dysfunction **(Williams 2012)**. Hence, unraveling the molecular signaling pathways that regulate Agrp and POMC neuronal function could provide a better understanding of the molecular mechanisms which regulate feeding behavior and energy homeostasis and could in extension lead to the development of novel anti-obesity treatments. Due to the important role of mTORC1 as a nutrient and energy sensor, mTORC1 could potentially be involved in the regulation of Agrp and POMC neuronal function. Indeed, POMC-specific hyper-activation of mTORC1 signaling leads to hyperphagia-induced obesity due to impaired POMC expression **(Mori et al. 2009)**. This indicates that mTORC1 signaling is crucial for POMC function. However, the role of mTORC1 signaling in Agrp neurons has so far not been thoroughly examined.

Thus, in the first part of this thesis we focused on elucidating the role of mTORC1 signaling in Agrp neurons of the hypothalamus. To this end we generated Agrp neuron-specific *raptor* (Agrp-*raptor*) KO mice to inactivate mTORC1 signaling specifically in Agrp neurons and analyzed the systemic metabolic phenotype of these mice. With this project we wanted to answer the following questions:

1. Does inactivation of mTORC1 signaling in Agrp neurons alter feeding behavior and/or systemic energy homeostasis?
2. How do Agrp-*raptor* KO mice react to a dietary challenge, such as HFD feeding?
3. Is mTORC1 signaling involved in the regulation of orexigenic neuropeptide expression?

In the second part of this thesis we investigated whether mTORC2 signaling in adipose tissue regulates non-shivering thermogenesis (NST) and thereby affects systemic energy homeostasis. Activation of NST and subsequent energy dissipation has been proposed as an alternative strategy to treat obesity and decrease the risk for obesity-associated diseases **(Clapham and Arch 2011)**. Additionally, cold-induced glucose uptake into BAT through an insulin-independent mechanism could provide

a promising therapy to decrease blood glucose in insulin-resistant, diabetic patients. Thus, identifying novel molecular pathways involved in the regulation of NST could help develop novel therapies for obesity and diabetes. Since mTORC2 signaling is an important regulator of insulin-stimulated glucose uptake and glycolysis, we hypothesized that mTORC2 signaling in BAT could be involved in the regulation of cold-induced glucose uptake and temperature homeostasis. To test this hypothesis, we used AdRiKO mice, where mTORC2 signaling is specifically inactivated in mature adipocytes, and investigated how these mice responded to thermogenic stimuli, such as cold exposure or β-adrenergic stimulation. With this second project we wanted to answer the following questions:

1. How is mTORC2 signaling in BAT and WAT regulated upon cold exposure or β-adrenergic stimulation?
2. Does inactivation of mTORC2 signaling in adipose tissue affect body temperature and/or cold tolerance?
3. Is mTORC2 signaling in BAT involved in cold-induced glucose uptake?

With these two projects we hope to provide a better understanding of how mTOR signaling in a particular tissue can affect whole-body metabolism and thereby influence systemic energy homeostasis. This knowledge could potentially lead to new drug targets for anti-obesity therapies.

Chapter 3: Results

mTORC1 signaling in Agrp neurons mediates circadian expression of Agrp and NPY but is dispensable for regulation of feeding behavior

Verena Albert, Marion Cornu and Michael N. Hall*

Biozentrum, University of Basel, Klingelbergstrasse 50/70 4056 Basel, Switzerland

*Correspondence: m.hall@unibas.ch, +41 61 267 21 50

(Submitted to Biochemical and Biophysical Research Communications)

Abstract

Orexigenic agouti-related protein/neuropeptide Y (Agrp/NPY) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons of the hypothalamus regulate feeding behavior and energy homeostasis. An understanding of the molecular signaling pathways that regulate Agrp/NPY and POMC function could lead to novel treatments for metabolic disorders. Target of Rapamycin Complex 1 (TORC1) is a nutrient activated protein kinase and central controller of growth and metabolism. We therefore investigated the role of mammalian TORC1 (mTORC1) in Agrp neurons. We generated and characterized Agrp neuron-specific *raptor* knockout (Agrp-*raptor* KO) mice. Agrp-*raptor* KO mice displayed reduced, non-circadian expression of *Agrp* and *NPY* but normal feeding behavior and energy homeostasis on both normal and high fat diet. Thus, mTORC1 in Agrp neurons controls circadian expression of orexigenic neuropeptides but is dispensable for the regulation of feeding behavior and energy metabolism.

Keywords: mTORC1; raptor; hypothalamus; Agrp; NPY; metabolism

Introduction

The arcuate nucleus (ARC) of the hypothalamus mediates whole body energy homeostasis including feeding behavior. It contains orexigenic agouti-related protein/neuropeptide Y (Agrp/NPY) neurons that secrete the neuropeptides Agrp and NPY, and anorexigenic pro-opiomelanocortin (POMC) neurons that secrete the neuropeptide POMC. Agrp/NPY neurons induce feeding and decrease energy expenditure while POMC neurons inhibit feeding and increase energy expenditure **(Cone et al. 2001, Neary et al. 2004, Arora and Anubhuti 2006)**. The ARC receives input from peripheral organs via hormones, such as insulin from the pancreas, leptin from adipose tissue and ghrelin from the intestine **(Woods 2009)**. Thus, dysfunction of Agrp/NPY and POMC neurons can contribute to the development of obesity **(Velloso et al. 2009)**. For example, Agrp/NPY and POMC neurons of obese patients often develop resistance to leptin and insulin, leading to altered feeding behavior and disturbed energy metabolism **(Williams 2012)**. It is therefore important to

understand the signaling pathways that regulate Agrp/NPY and POMC function.

The mammalian Target of Rapamycin (mTOR) signaling pathway integrates energy and nutrient levels to regulate cellular and organismal growth and metabolism **(Laplante and Sabatini 2012, Dibble and Manning 2013, Albert and Hall 2014, Shimobayashi and Hall 2014)**. mTOR forms two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2 **(Wullschleger et al. 2006)**. mTORC1 is activated by growth factors, amino acids and cellular energy. Activation of mTORC1 by growth factors is mediated via the PI3K-PDK1-Akt signaling pathway. mTORC1 signaling stimulates protein synthesis, nucleotide biosynthesis, and lipogenesis, while it inhibits autophagy **(Dibble and Manning 2013, Shimobayashi and Hall 2014)**. Due to its central role in cell growth and metabolism, deregulation of mTORC1 signaling is often associated with the development of metabolic disorders, such as diabetes and obesity **(Dazert and Hall 2011)**. Several studies have implicated hypothalamic mTORC1 signaling in the regulation of energy homeostasis and food intake. For example, feeding status regulates mTORC1 activity in both Agrp/NPY and POMC neurons **(Cota et al. 2006)**, and hyperactivation of mTORC1 in POMC neuron leads to hyperphagia-induced obesity **(Mori et al. 2009)**.

To further examine the role of mTORC1 signaling in the ARC, we generated Agrp neuron-specific *raptor* knockout (Agrp-*raptor* KO) mice and assessed whole body energy metabolism, feeding behavior and adaptation to metabolic stress. Ablation of mTORC1 signaling in Agrp neurons resulted in reduced, non-circadian expression of orexigenic neuropeptides. However, this did not affect *ad libitum* food intake or ghrelin- or fasting-induced feeding. Furthermore, neither male nor female Agrp-*raptor* KO mice displayed changes in whole-body metabolism compared to control mice. On both normal and high fat diet (HFD), Agrp-*raptor* KO mice displayed similar body weight, glucose homeostasis and whole-body metabolic rates compared to control mice. Hence, mTORC1 signaling in Agrp neurons is dispensable for regulation of whole-body metabolism and feeding behavior.

Material and Methods

Animals. Agrp-*raptor* KO mice were generated by crossing Agrp-IRES-Cre mice **(Tong et al. 2008)** with *raptor*$^{LoxP/LoxP}$ mice **(Polak et al. 2008)**. For immunohistochemistry Agrp-IRES-Cre or Agrp-*raptor* KO mice were crossed with Rosa26-STOP $^{LoxP/LoxP}$-EGFP mice **(Mao et al. 2001)** to obtain mice with EGFP expression in Agrp neurons. Mice were housed at 22°C in a conventional facility with a 12h light/12h dark cycle. The standard diet contained 15.8kcal% of fat (Promivi Kliba). The high fat diet contained 60kcal% of fat (Harlan). All experiments were performed in accordance with the federal guidelines for animal experimentation and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt.

Locomotor Activity, Metabolic Rate, and Food Intake. Locomotor activity, metabolic rate, and food intake was measured for 48h using a comprehensive laboratory animal monitoring system (CLAMS, Linton Instrumentation and Columbus Instruments) after 24h of acclimatization.

Glucose Tolerance Test. Mice were starved for 6h and subsequently injected I.P. with glucose (2g/kg). Blood glucose was measured in tail vain blood using a glucose meter (Accu-Chek, Roche).

Microdissection of ARC. ARC was isolated from brains by micropunch **(Palkovits 1973)**. Brains were sliced in 0.5mm thick coronal sections with a rodent brain slicer matrix (Zivic Instruments). Sections containing the ARC were identified and ARC was excised with a hollow needle (1.25mm diameter).

RNA Isolation and RT-PCR. Total RNA from hypothalamus or ARC was isolated with SV total RNA isolation kit (Promega) followed by cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Semi-quantitative real-time PCR analysis was performed using fast SYBR green (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels were determined by normalizing to *TBP* expression using the ΔΔCt method.

Primers used:
TBP fw: TGCTGTTGGTGATTGTTGGT
TBP rv: CTGGCTTGTGTGGGAAAGAT
Agrp fw: AACCTCTGTAGTCGCACCTAGC
Agrp rv: AAACCGTCCCATCCTTTATTCT
NPY fw: AGGCTTGAAGACCCTTCCAT
NPY rv: GATGAGGGTGGAAACTTGGA
POMC fw: GAGCTGATGACCTCTAGCCTCT
POMC rv: ATCAGAGCCGACTGTGAAATCT.

Protein Isolation and Western Blot. Hypothalamus or ARC tissue was homogenized in lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1% Triton X-100, protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich)). Proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Whatman). Antibodies used: Akt (Santa Cruz), Akt-pS473, Akt-pT308, S6, S6-pS235/236, 4E-BP1, 4EBP1-pS37/48 (all Cell Signaling), actin (Millipore)

Recombination. Genomic DNA was isolated by incubating the tissues in PBND buffer containing 0.1mg Proteinase K at 57°C over night, followed by proteinase K inactivation at 95°C for 10 minutes. PCR was performed for Cre or for the genomic region flanking the floxed sites in the *raptor* gene to determine *raptor* deletion. Primers used: Cre fw: TGTGGCTGATGATCCGAATA; Cre rv: GCTTGCATGATCTCCGGTAT; *raptor* deletion fw: ATGGTAGCAGGCACACTCTTCATG; *raptor* deletion rv: CTCAGAGAACTGCAGTGCTGAAGG.

Immunostaining. Mice were transcardially perfused with 4% paraformaldehyde, followed by over night fixation of the brain in 4% paraformaldehyde. Brains were dehydrated, embedded in paraffin and cut in 5um thick coronal sections. Antibodies used: GFP (Abcam) and pS6 S235/236 (Cell Signaling). DAPI was used to stain the nuclei.

Blood Analysis. Blood glucose was measured in tail vain blood using a

glucose meter (Accu-Chek, Roche).

Fasting refeeding. Mice were starved over night prior to the experiment. At ZT2 mice were refed and food was weighted at the indicated times after refeeding.

Ghrelin stimulation. Mice were fed ad libitum and injected I.P. with 1mg/kg of ghrelin or vehicle. Food intake was measured 30 minutes after ghrelin administration.

Results

Deletion of *raptor* in Agrp neurons leads to inactivation of mTORC1 signaling. To investigate the role of mTORC1 signaling in Agrp neurons, we generated Agrp-*raptor* KO mice by crossing Agrp-IRES-Cre mice **(Tong et al. 2008)** with *raptor*$^{LoxP/LoxP}$ mice **(Polak et al. 2008)**. To assess the tissue specificity of the knockout, we performed PCR to monitor *raptor* ablation. Excision of the floxed *raptor* allele was detected in the ARC of Agrp-*raptor* KO mice while the hypothalamic region surrounding the ARC and all other organs tested revealed an intact *raptor* gene **(Figure 1A)**. Thus, *raptor* ablation was specific to the ARC. Next, we investigated PI3K-mTORC1 signaling in the ARC of control and Agrp-*raptor* KO mice in a fasting-refeeding paradigm. In the ARC of control mice, feeding induced phosphorylation of Akt at S473 and of the mTORC1 target S6 at S235/236 **(Figure 1B)**. These findings demonstrate that the PI3K-mTORC1 signaling pathway is activated in the ARC upon feeding, as shown previously **(Cota et al. 2006)**. In contrast, phosphorylation of S6 at S235/236 was absent in the ARC of Agrp-*raptor* KO mice, indicating that mTORC1 signaling was indeed inactive upon knockout of *raptor* **(Figure 1B)**. In line with previous studies using *raptor*-deficient mouse models **(Bentzinger et al. 2008, Polak et al. 2008)**, Agrp-*raptor* KO mice displayed hyper-phosphorylation of Akt-S473 and -T308 in the ARC **(Figure 1B)**, due to absence of the S6K-mediated negative feedback loop **(Harrington et al. 2004, Shah et al. 2004, Um et al. 2004, Harrington et al. 2005)**. To assess mTORC1 signaling specifically in Agrp neurons, we immunostained phospho-S6 in coronal brain sections of Agrp-*raptor* KO and

control mice expressing EGFP specifically in Agrp neurons. In line with our previous findings **(Figure 1B)**, S6 phosphorylation was induced in Agrp neurons upon refeeding in control mice **(Figure 1C)**, but was absent in Agrp neurons of Agrp-*raptor* KO mice. Thus, as expected, mTORC1 signaling was defective in Agrp neurons upon raptor deletion.

Inactivation of mTORC1 signaling in Agrp neurons does not affect whole-body metabolism. To investigate the role of mTORC1 signaling in Agrp neurons on whole-body metabolism, we measured body weight, body composition, glucose tolerance, metabolic rate and locomotor activity in young (3 months old) and old (1 year old) Agrp-*raptor* KO and control mice. Surprisingly, Agrp-*raptor* KO mice did not display any differences in body weight **(Figure 2A)**, body composition **(Figure 2B)** or glucose tolerance **(Figure 2C)** compared to control mice at either age. In the aged group, Agrp-*raptor* KO mice showed a trend toward higher body weight compared to control littermates **(Figure 2A)**. However, this difference did not reach statistical significance. Moreover, there was no difference in oxygen consumption **(Figure 2D)**, carbon dioxide production **(Figure 2E)**, respiratory exchange ratio **(Figure 2F)** and locomotor activity **(Figure 2G)** in Agrp-*raptor* KO mice compared to control mice. Taken together, these findings demonstrate that inactivation of mTORC1 signaling in Agrp neurons does not affect whole-body metabolism in either young or aged mice.

Agrp-*raptor* KO mice display impaired circadian rhythm of Agrp and NPY expression but not altered feeding behavior. Since Agrp neurons regulate feeding behavior in a circadian manner, we investigated food intake, mTORC1 signaling, and *Agrp* and *NPY* mRNA expression levels over a 24h cycle in Agrp-*raptor* KO and control mice fed *ad libitum*. Mice were sacrificed at Zeitgeber 0 (ZT0), ZT12, ZT18 and ZT24. At ZT0, the lights turn on, ZT12 marks when the light turns off. Similar to other organs **(Cao et al. 2011, Cornu et al. 2014, Khapre et al. 2014)**, phosphorylation of the mTORC1 downstream targets S6 (S235/236) and 4E-BP1 (T37/48) was regulated in a circadian manner in the ARC of control mice. mTORC1 signaling activity in the ARC peaked at ZT18, during the dark phase when mice are active and eat

(Figure 3A). In Agrp-*raptor* KO mice, phosphorylation of S6 and 4E-BP1 was undetectable in the ARC at all time points, confirming that mTORC1 signaling was ablated **(Figure 3A)**. Importantly, mRNA expression of both *Agrp* and *NPY* also oscillated in a circadian manner similar to mTORC1 signaling activity **(Figure 3B)**. Similar to S6 (S235/236) and 4E-BP1 (T37/48) phosphorylation, *Agrp* and *NPY* expression was highest at ZT18 **(Figure 3B)**. Interestingly, expression of both *Agrp* and *NPY* was reduced and non-oscillating in Agrp-*raptor* KO mice, leading to a significant reduction in *Agrp* and *NPY* levels at ZT18 **(Figure 3B)**. Hence, the circadian pattern of mTORC1 signaling in the ARC might regulate the circadian oscillation of *Agrp* and *NPY* expression. In contrast to this, *POMC* expression was not circadian and did not differ between the genotypes **(Figure 3B)**. Thus, inactivation of mTORC1 in Agrp neurons does not lead to compensatory alterations in *POMC* levels. Surprisingly, despite a significant decrease in *Agrp* and *NPY* mRNA levels, Agrp-*raptor* KO mice did not display a decrease in *ad libitum* food intake compared to controls **(Figure 3C)**.

Since *Agrp* and *NPY* expression was reduced in Agrp-*raptor* KO mice, we hypothesized that induction of *Agrp* and *NPY* expression might also be impaired in starved KO mice. To test this, we measured *Agrp* and *NPY* mRNA in Agrp-*raptor* KO and control mice that were starved overnight or fed *ad libitum*. Unlike the decreased circadian *Agrp* and *NPY* expression, Agrp-*raptor* KO mice were able to induce both *Agrp* and *Npy* mRNA expression upon fasting **(Figure 3D)**. Hence, induction of *Agrp* and *NPY* mRNA upon fasting is likely mediated by an mTORC1-independent mechanism. In line with the unchanged induction of *Agrp* and *NPY* expression upon starvation, Agrp-*raptor* KO mice displayed similar feeding in response to a fasting-refeeding challenge as compared to control mice. We fasted Agrp-*raptor* KO and control mice overnight and measured food intake after refeeding in the morning. Agrp-*raptor* KO mice consumed a similar amount of food compared to control mice at all time points after refeeding **(Figure 3E)**. Next, we investigated whether mTORC1 signaling is required for induction of feeding upon ghrelin stimulation. Ghrelin is an orexigenic neuropeptide that is secreted upon starvation from ghrelin-producing cells in the gastrointestinal tract and is able to activate Agrp neurons **(Nakazato et al. 2001, Wren et al.**

2001). We injected fed Agrp-*raptor* KO and control mice with ghrelin and measured food intake. Similar to our results above, Agrp-*raptor* KO mice again failed to display a defect in food intake **(Figure 3F)**. The above taken together indicates that feeding behavior is not impaired in Agrp-*raptor* KO mice.

Agrp-*raptor* KO mice respond to metabolic stress. Chronic, excessive consumption of nutrients, in particular fat, is a metabolic stress that induces compensatory metabolic alterations. Since Agrp-*raptor* KO mice failed to display metabolic alterations when fed a standard diet, we investigated the response of Agrp-*raptor* KO mice to metabolic stress. To induce metabolic stress, we fed Agrp-*raptor* KO and control mice a high fat diet (HFD) (60kcal% from fat) for 10 weeks. Similar to the results obtained on a normal diet, Agrp *raptor*-KO mice displayed similar weight gain compared to controls (Figure 4A). Moreover, body composition and food intake did not significantly differ between the genotypes **(Figure 4B and 4C)**. To assess glucose tolerance in HFD-fed Agrp-*raptor* KO mice we performed an intraperitoneal glucose tolerance test (GTT) and measured blood glucose in both fasted and fed animals. While HFD feeding led to impaired glucose tolerance in control and Agrp-*raptor* KO mice, no difference in glucose clearance rates could be detected between genotypes **(Figure 4D)**. Furthermore, control and Agrp-*raptor* KO mice displayed similar blood glucose levels upon HFD feeding **(Figure 4E)**. Finally, we measured oxygen consumption **(Figure 4F)**, carbon dioxide production **(Figure 4G)**, respiratory exchange ratio **(Figure 4H)**, and locomotor activity **(Figure 4I)** in Agrp-*raptor* KO and control mice fed a HFD. In line with our results obtained with mice on a normal diet, HFD-fed Agrp-*raptor* KO mice failed to display any alteration in these parameters as compared to control mice. Taken together, these findings demonstrate that ablation of mTORC1 signaling in Agrp neurons does not alter the response to metabolic stress such as that caused by a HFD.

Discussion

mTOR is a nutrient sensor. Consequently, mTORC1 signaling in the hypothalamus has been suggested to be involved in the regulation of whole

body energy homeostasis and feeding behavior. Indeed, hyper-activation of mTORC1 signaling specifically in POMC neurons through knockout of *TSC1* results in hyperphagia-induced obesity **(Mori et al. 2009)**. These findings demonstrate a role for mTORC1 signaling in POMC neurons in the regulation of feeding behavior. However, the role of mTORC1 signaling in Agrp neurons has so far not been thoroughly examined. To elucidate the role of mTORC1 signaling in these neurons, we generated Agrp-*raptor* KO mice. Surprisingly, Agrp-*raptor* KO mice displayed no impairment in whole-body energy metabolism, glucose homeostasis and feeding behavior, both on normal and high fat diet. Hence, mTORC1 signaling in Agrp neurons is dispensable for the regulation of energy balance and feeding behavior. This is in agreement with a recent publication demonstrating that hyper-activation of mTORC1 in Agrp neurons, through deletion of *TSC1*, did not affect feeding behavior or energy homeostasis **(Yang et al. 2012)**. Thus, disruption of mTORC1 signaling in POMC neurons seemingly affects whole-body energy homeostasis, whereas perturbation of mTORC1 signaling in Agrp neurons does not. Interestingly, similar to mTORC1, Agrp-specific inactivation of mTORC2 signaling through deletion of *rictor* did not result in any alterations in feeding behavior and energy homeostasis, while POMC-specific deletion of *rictor* caused hyperphagia-induced obesity **(Kocalis et al. 2014)**.

These findings are in line with the observation that perturbation of POMC neuronal function has a more profound effect on feeding behavior and energy metabolism as compared to perturbation of Agrp/NPY neuronal function. For example, inhibition of *Agrp* or *NPY* expression does not affect feeding behavior **(Erickson et al. 1996, Marsh et al. 1998, Palmiter et al. 1998, Qian et al. 2002)**. In contrast, impaired *POMC* expression or POMC neuronal function results in hyperphagia, obesity and disturbance in energy homeostasis **(Krude et al. 1999, Yaswen et al. 1999)**. While depletion of Agrp neurons in neonates only mildly affects feeding behavior, ablation of Agrp neurons in adult mice results in a strong decrease in food intake, leading to starvation **(Luquet et al. 2005)**. These findings imply that the requirement for Agrp neurons for the regulation of feeding can be circumvented when their function is impaired early in life. A limitation of the Agrp-*raptor* KO mice used in this study is therefore that mTORC1 signaling is inactivated in Agrp

neurons already in the neonate state. It could thus be that compensatory mechanisms have developed to circumvent the requirement for mTORC1 signaling for Agrp neuronal function in Agrp-*raptor* KO mice. Future studies should address whether disruption of mTORC1 signaling in Agrp neurons in adult mice impacts energy homeostasis and feeding behavior.

We found that Agrp-*raptor* KO mice display impaired circadian expression of *Agrp* and *NPY* but normal feeding behavior. Interestingly, mTORC1 activity in the ARC of control mice was highest during the dark phase, similar to the expression pattern of *Agrp* and *NPY* mRNA. Since Agrp-*raptor* KO mice were unable to induce mTORC1 signaling in Agrp neurons during the dark phase, it is likely that circadian mTORC1 signaling regulates circadian *Agrp* and *NPY* mRNA expression. In contrast to the defective *Agrp* and *NPY* expression observed in *ad libitum* fed mice, Agrp-*raptor* KO mice strongly induced *Agrp* and *NPY* mRNA expression in response to overnight starvation as observed in control mice. Importantly, mTORC1 activity was low in the ARC of control mice after overnight starvation. These finding suggest that the induction of *Agrp* and *NPY* mRNA expression after overnight starvation is mTORC1-independent and thus mechanistically different compared to the circadian regulation of *Agrp* and *NPY* mRNA expression. Future studies should be aimed at identifying the molecular basis for this difference in the regulation of *NPY* and *Agrp* expression.

Acknowledgements

We acknowledge support from the Swiss National Science Foundation, the Louis-Jeantet Foundation, the Werner Siemens Foundation (VA), and the Canton of Basel.

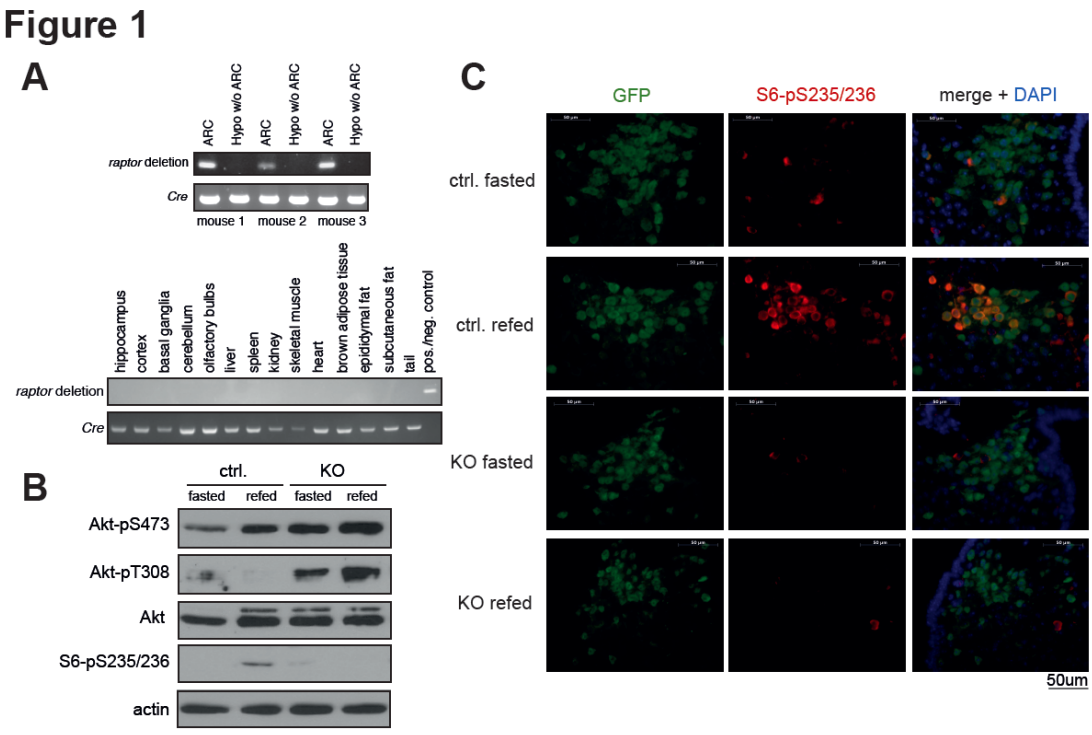


Figure 1. *Deletion of raptor in Agrp neurons leads to inactivation of mTORC1 signaling.* (A) PCR for *Cre* and *raptor* deletion in the indicated organs from Agrp-*raptor* KO mice. (B) Immunoblot of protein lysates from the ARC of Agrp-*raptor* KO and control mice with the indicated antibodies. (C) Immunostaining of coronal brain sections from Agrp-*raptor* KO reporter mice and control reporter mice for EGFP to visualize Agrp neurons and S6-pS235/236 to visualize mTORC1 activity.

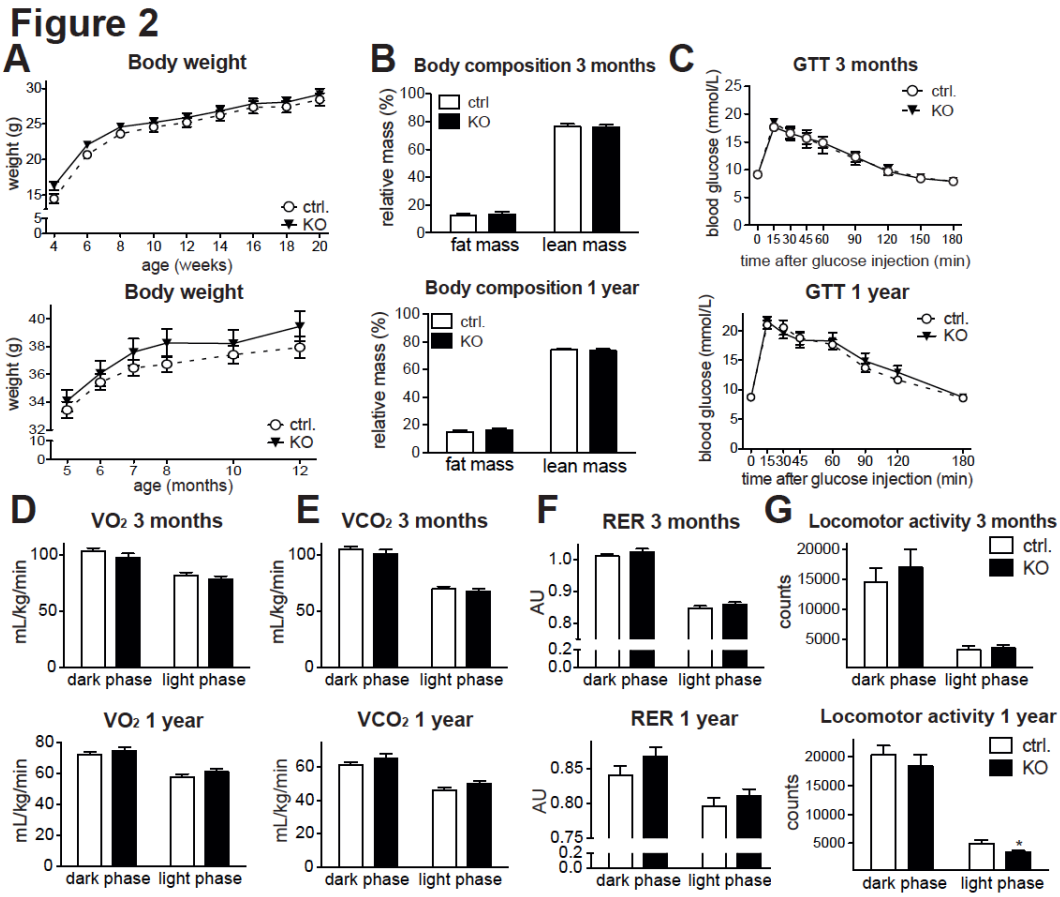


Figure 2. *Inactivation of mTORC1 signaling in Agrp neurons does not affect whole-body metabolism.* (A) Body weight of young and old Agrp-*raptor* KO and control mice. (B) Body composition of young and old Agrp-*raptor* KO and control mice. (C) GTT of young and old Agrp-*raptor* KO and control mice. (D) Oxygen consumption (VO_2) of young and old Agrp-*raptor* KO and control mice. (E) Carbon dioxide production (VCO_2) of young and old Agrp-*raptor* KO and control mice. (F) Respiratory exchange ratio (RER) of young and old Agrp-*raptor* KO and control mice. (G) Locomotor activity of young and old Agrp-*raptor* KO and control mice. Data represent mean ± SEM. Statistically significant differences between Agrp-*raptor* KO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001).

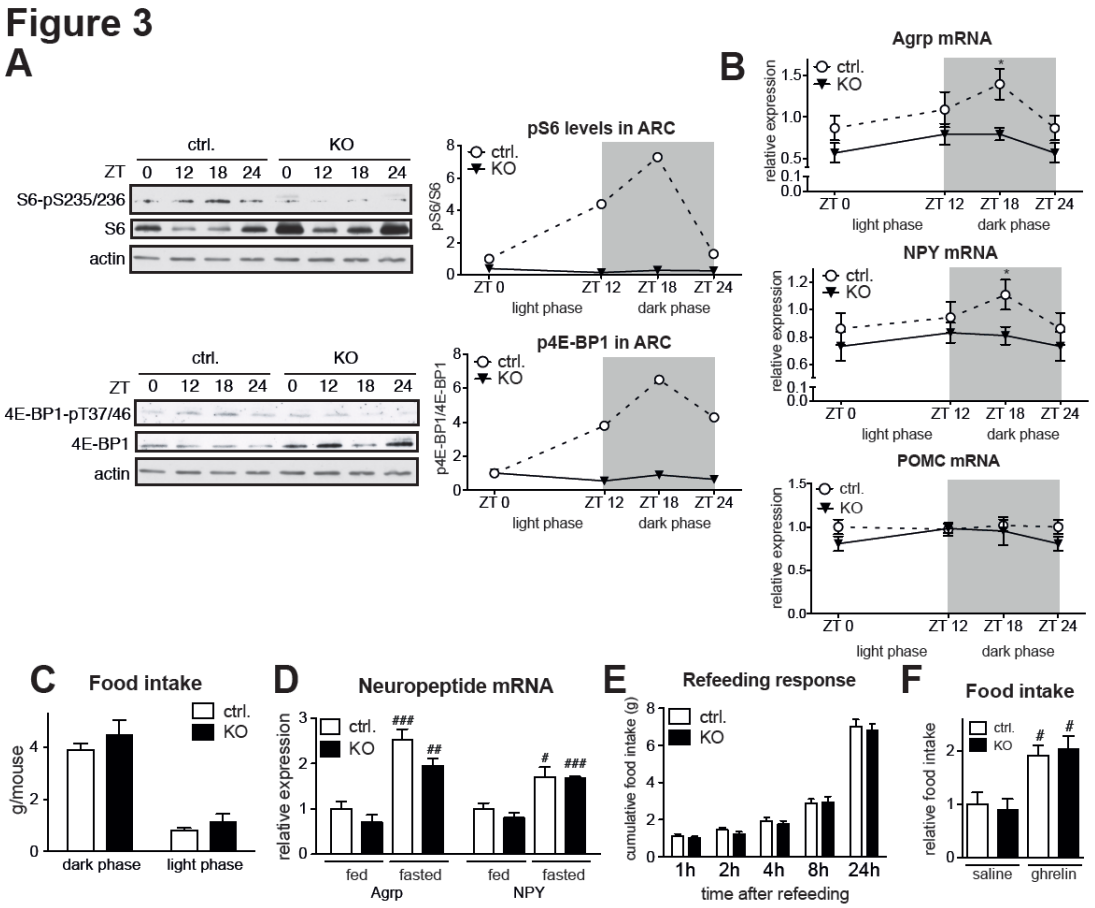


Figure 3. *Agrp-raptor KO mice display impaired circadian rhythm of Agrp and NPY expression but not altered feeding behavior.* (A) Immunoblot analysis with the indicated antibodies of protein lysates from the ARC of Agrp-*raptor* KO and control mice sacrificed at the indicated time points. (B) qRT-PCR analysis of *Agrp*, *NPY* and *POMC* mRNA in the hypothalamus of Agrp-*raptor* KO and control mice sacrificed at the indicated time points. (C) *Ad libitum* food intake of Agrp-*raptor* KO and control mice. (D) qRT-PCR analysis of *Agrp* and *NPY* mRNA in the hypothalamus of Agrp-*raptor* KO and control mice fed ad libitum or starved over night. (E) Refeeding response of Agrp-*raptor* KO and control mice after over night starvation. (H) Feeding response of Agrp-*raptor* KO and control mice after ghrelin injection. Data represent mean ± SEM. Statistically significant differences between Agrp-*raptor* KO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001). Statistically significant differences between feeding conditions or ghrelin stimulation are indicated with a number sign (#=p<0.05; ##=p<0.01; ###=p<0.001).

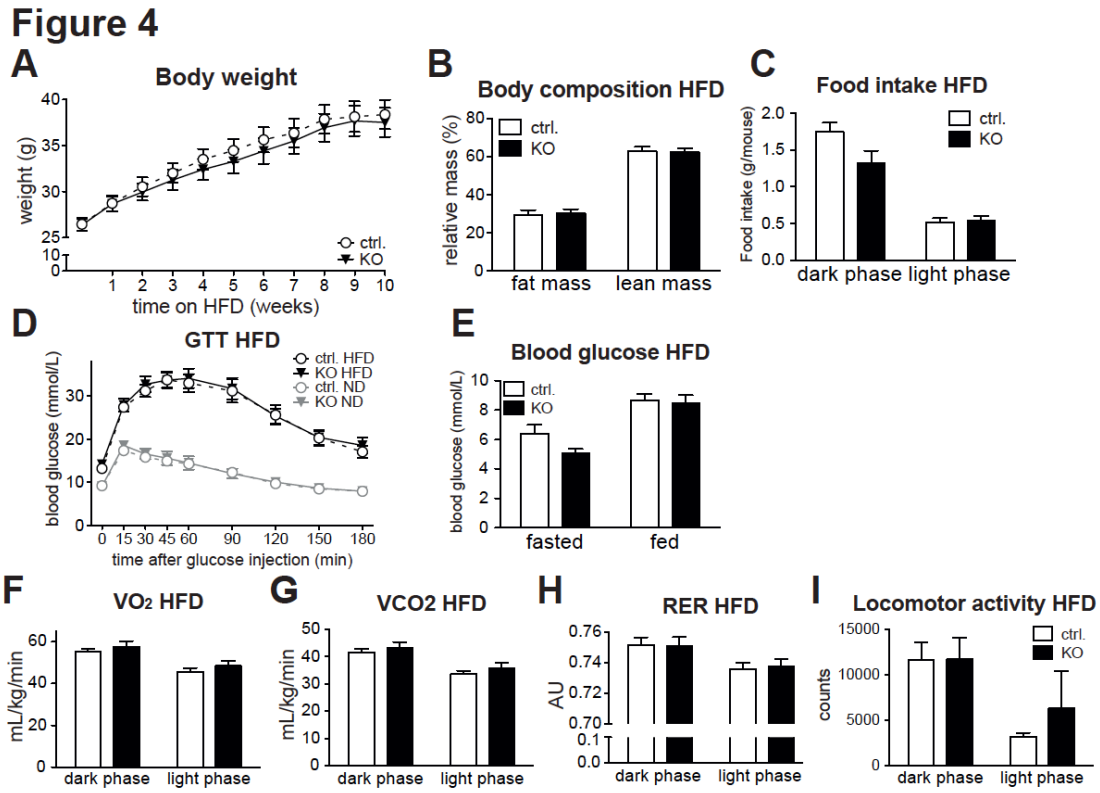


Figure 4. *Agrp-raptor KO mice respond to metabolic stress.* (A) Body weight of HFD fed Agrp-*raptor* KO and control mice. (B) Body composition of HFD fed Agrp-*raptor* KO and control mice. (C) Food intake of HFD fed Agrp-*raptor* KO and control mice. (D) GTT of HFD fed Agrp-*raptor* KO and control mice. (E) Fasted and fed ad libitum blood glucose levels of HFD fed Agrp-*raptor* KO and control mice. (F) Oxygen consumption (VO_2) of HFD fed Agrp-*raptor* KO and control mice. (G) Carbon dioxide production (VCO_2) of HFD fed Agrp-*raptor* KO and control mice. (H) Respiratory exchange ratio (RER) of HFD fed Agrp-*raptor* KO and control mice. (I) Locomotor activity of HFD fed Agrp-*raptor* KO and control mice. Data represent mean ± SEM.

mTORC2 sustains thermogenesis via Akt-induced glucose uptake and glycolysis in BAT

Verena Albert[a], Kristoffer Svensson[a], Mitsugu Shimobayashi[a], Veronica Jimenez[b], Sergio Muñoz[b], Christoph Handschin[a], Fatima Bosch[b], Michael N. Hall[a]*

Biozentrum, University of Basel, 4056 Basel, Switzerland[a]
Center of Animal Biotechnology and Gene Therapy, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain [b]

*Correspondence: m.hall@unibas.ch

Abstract

Activation of non-shivering thermogenesis (NST) in brown adipose tissue (BAT) has been proposed as an anti-obesity treatment. Moreover, cold-induced glucose uptake could normalize blood glucose levels in insulin resistant patients. It is therefore important to identify novel regulators of NST and cold-induced glucose uptake. Mammalian Target of Rapamycin Complex 2 (mTORC2) mediates insulin-stimulated glucose uptake in metabolic tissues but its role in NST is unknown. We show that mTORC2 is activated in brown adipocytes upon β-adrenergic stimulation. Furthermore, mice lacking mTORC2 specifically in adipose tissue (AdRiKO mice) are hypothermic, display increased sensitivity to cold, and show impaired cold-induced glucose uptake and glycolysis. Restoration of glucose uptake in BAT by overexpression of hexokinase II or activated Akt2 was sufficient to increase body temperature and improve cold tolerance in AdRiKO mice. Thus, mTORC2 in BAT mediates temperature homeostasis via regulation of cold-induced glucose uptake.

Keywords: brown adipose tissue, thermogenesis, mTORC2, glucose uptake

Introduction

Non-shivering thermogenesis (NST) in brown adipose tissue (BAT) allows mammals to maintain stable body temperature in a cold environment. Upon cold exposure, norepinephrine (NE) is released from sympathetic nerves and binds to β_3-adrenergic receptors on brown adipocytes to induce NST. β_3-adrenergic receptor stimulation induces cAMP production and subsequent induction of lipolysis, β-oxidation and mitochondrial uncoupling **(Cannon and Nedergaard 2004)**. Mitochondrial uncoupling occurs through activation of uncoupling protein 1 (UCP1). UCP1 is a mitochondrial transmembrane protein specifically expressed in brown adipocytes and brown-like, beige adipocytes. Once activated, UCP1 dissipates the proton gradient across the inner mitochondrial membrane generated by the electron transport chain. This uncouples proton flux into the mitochondria from ATP production, resulting in heat generation **(Klaus et al. 1991, Busiello et al. 2015)**. To

compensate for the loss of mitochondrial ATP production due to uncoupling, β-adrenergic stimulation enhances glucose uptake and glycolysis in BAT **(Greco-Perotto et al. 1987, Vallerand et al. 1990, Hao et al. 2015)**. Due to the ability of BAT to burn energy efficiently and to reduce blood glucose levels, activation of NST has been proposed as an alternative strategy for weight loss in obese patients **(Cypess et al. 2009, van Marken Lichtenbelt et al. 2009, Virtanen et al. 2009, Clapham and Arch 2011)** and for normalization of blood glucose levels in insulin resistant diabetic patients. Thus, identifying novel regulators of NST could provide new drug targets for anti-obesity and diabetes treatments.

The mammalian target of rapamycin (mTOR) signaling network is a central regulator of cell growth and metabolism **(Laplante and Sabatini 2012, Dibble and Manning 2013, Albert and Hall 2014, Shimobayashi and Hall 2014)**. mTOR is a highly conserved protein kinase found in two structurally and functionally distinct complexes named mTOR complex 1 (mTORC1) and mTORC2. mTORC1 is sensitive to the macrolide rapamycin, and contains mTOR, mammalian lethal with sec-13 protein (mLST8), and regulatory associated protein of mTOR (raptor). mTORC2 is rapamycin-insensitive, contains mTOR, mLST8, mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and rapamycin insensitive companion of mTOR (rictor). mTORC2 is activated by growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), via phosphatidylinositol 3-kinase (PI3K)-dependent ribosome association **(Zinzalla et al. 2011)**. mTORC2 downstream targets are members of the AGC kinase family, such as Akt, serum/glucocorticoid regulated kinase (SGK) and protein kinase C (PKC) **(Sarbassov et al. 2005, Jacinto et al. 2006, Garcia-Martinez and Alessi 2008, Ikenoue et al. 2008, Cybulski and Hall 2009)**, through which mTORC2 promotes lipogenesis, glucose uptake, glycolysis, and cell survival **(Manning and Cantley 2007, Kumar et al. 2008, Hagiwara et al. 2012, Yuan et al. 2012)**. Due to its role in mediating lipid and glucose homeostasis, dysfunction of mTORC2 signaling has been implicated in the development of insulin resistance and diabetes. However, a role for mTORC2 in thermogenesis, and in particular NST, has so far not been investigated.

Here we show that β-adrenergic stimulation and cold exposure activate mTORC2 signaling in brown adipocytes *in vitro* and *in vivo*. We find that mTORC2 in BAT stimulates cold-induced glucose uptake and glycolysis. Consequently, mice with adipose tissue-specific inactivation of mTORC2 (AdRiKO mice) are hypothermic and unable to maintain stable body temperature upon cold exposure. Restoration of either Akt signaling or glucose metabolism in BAT of AdRiKO mice restored body temperature and improved cold tolerance. Thus, mTORC2 in BAT is essential for maintenance of energy homeostasis and body temperature upon cold exposure.

Material and Methods

Cell Culture. SV40T-immortalized C57/B6 mouse brown pre-adipocytes were kindly provided by Professor Johannes Klein (Lübeck, Germany) **(Klein et al. 2002)**. Preadipocytes were grown to confluency in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 20% fetal calf serum (FCS), 4.5g/L glucose, 2mM glutamine, 20nmol/L insulin, and 1nmol/L triiodothyronine. 24h after reaching confluency, adipocyte differentiation was induced by addition of 250umol/L indomethacin, 500umol/L isobutylmethylxanthine and 2ug/mL dexamethasone to the medium for 24h. Cell culture was continued for five more days before experiments were performed.

Animals. Adipose tissue-specific rictor knockout mice (AdRiKO) were already described and characterized previously **(Cybulski et al. 2009)**. Mice were housed at 22°C in a conventional facility with a 12h light/ 12h dark cycle. For all experiments male aP2-Cre; rictor$^{LoxP/LoxP}$ (AdRiKO) and rictor$^{LoxP/LoxP}$ (control) mice between 10-14 weeks of age were used. All experiments were performed in accordance with the federal guidelines for animal experimentation and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt.

In vivo Norepinephrine Stimulation. Mice were starved for 12h over night prior to norephinephrine administration. Mice were injected intraperitoneally

with 1mg/kg norepinephrine and sacrificed 30 minutes later.

Cold Exposure. Mice were housed in single cages with free access to water at 4°C for a period of 2h, 4h, or 8h. For the 4h and 8h cold exposure, food was removed specifically during cold exposure. For the 2h cold exposure food was removed 12h prior to cold exposure and during cold exposure to allow assessment of mTORC2 signaling.

Thermoneutrality. Mice were housed in single cages with free access to water and food at 30°C for a period of 10h.

Locomotor Activity, Metabolic Rate, and Body Temperature. Locomotor activity and metabolic rate was measured in 30 minute-intervals for the indicated time using a comprehensive laboratory animal monitoring system (CLAMS, Linton Instrumentation and Columbus Instruments) after 24h of acclimatization. Mice had free access to food and water during the acclimatization period. For the measurement period food was removed. Body temperature was measured using a rectal thermometer (BAT-12, Physitemp).

Ex vivo Oxygen Consumption and Extracellular Acidification Rate. Oxygen consumption and extracellular acidification rate of BAT were measured using an XF24 extracellular flux analyzer (Seahorse Biosciences). Mice were housed in single cages at either 22°C or 4°C for 4h without food and subsequently BAT was collected and cut in approximately 0.5ug big pieces. Tissues were washed 3 times with Seahorse assay buffer supplemented with 25mM glucose, 2mM glutamine, 1mM sodium pyruvate adjusted to pH 7.4. Subsequently BAT pieces were placed in the center of a Seahorse XF24 islet capture microplate containing 675uL of Seahorse assay buffer. After 30 minutes incubation at 37°C without CO_2, oxygen consumption and extracellular acidification rate was measured 10 times and normalized to tissue weight. From each mouse 5 individual BAT pieces were used for measurement.

BAT Triglyceride and Free Fatty Acid Measurement. For the free fatty acid measurement, the crude lipid fraction was extracted from BAT using choroform:methanol (2:1). From this fraction the amount of free fatty acids was determined using a commercial kit (HR Series NEFA-HR(2)) and normalized to tissue weight. For triglyceride measurement, the crude lipid fraction was further purified on a solid-phase extraction column (UPTI-CLEAN NH2-S 100mg/1mL SPE Colums, Interchim). Subsequently amount of triglycerides was determined using a commercial kit (TG PAP BioMérieux) and normalized to tissue weight.

RNA Isolation and RT-PCR. Total RNA from BAT or sWAT was isolated with TRIzol reagent (Sigma) and RNeasy kit (Qiagen) followed by cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Semi-quantitative real-time PCR analysis was performed using fast SYBR green (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels were determined by normalizing to either *RPL0* or *TBP* expression using the ΔΔCt method. The sequence for the primers used in this study can be found in Supplementary Table 1.

mtDNA Copy Number Determination. Total DNA was isolated from BAT by incubating the tissue in PBND buffer containing 0.1mg Proteinase K at 57°C over night, followed by proteinase K inactivation at 95°C for 10 minutes. DNA was subsequently purified using a standard chloroform/phenol/isoamyl alcohol precipitation. mtDNA was determined in relation to the genomic DNA by qRT-PCR using primers against the *D-loop* region for mtDNA and against the single-copy nuclear gene *Ndufv1* for genomic DNA. The sequence for the primers used can be found in Supplementary Table 1.

Protein Isolation and Western Blot. For norepinephrine or 8-Br-cAMP stimulation and subsequent Western Blot analysis in cells, cells were starved for 16h in DMEM supplemented with 1% FCS, 4.5g/L glucose and 2mM glutamine. Cells were pre-treated for 30 minutes with DMSO, 100nM Rapamycin, 125nM Torin, 100nM Wortmannin, 10uM ESI-09, or for 2h with 20uM H89 and subsequently stimulated with 3uM of norepinephrine for 2h or

1mM 8-Br-cAMP for 30min unless indicated otherwise. Subsequently, cells were harvested in cold RIPA buffer containing 50mM Tris-HCl (pH 7.5), 2mM EDTA, 2mM EGTA, 150mM NaCl, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich). For protein isolation from adipose tissue, tissue was homogenized in lysis buffer containing 100mM Tris-HCl (pH 7.5), 2mM EDTA, 2mM EGTA, 1% Triton X-100, protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich). Protein concentration was determined by Bradford assay and equal amounts of protein were separated on SDS-PAGE followed by transfer onto a nitrocellulose membrane (Whatman). The following antibodies were used to detect the proteins of interest: Akt (Cell Signaling, cs-4685), Akt-pS473 (Cell Signaling, cs-9271), Creb (Cell Signaling, cs-9196), Creb-pS133 (Cell Signaling, cs-9197), actin (Millipore, MAB1501), PKA (Cell Signaling, cs-4782), PKA-pT192 (Cell Signaling, cs-4781), AMPK (Cell Signaling, cs-2532), AMPK-pT172 (Cell Signaling, cs-2535), raptor (Cell Signaling, cs-2280), raptor-pS792 (Cell Signaling, cs-2083), ACC (Cell Signaling, cs-3662), ACC-pS79 (Cell Signaling, cs-3661), perilipin (Cell Signaling, cs-9349), perilipin-pS497 (Vala Sciences, 4855), HSL (Cell Signaling, cs-4107), HSL-pS563 (Cell Signaling, cs-4139), PKCα (Cell Signaling, cs-2056), PKCα-pS638/641 (Cell Signaling, cs-9375), Mitoprofile (MitoSciences, MS604), HKI (Cell Signaling, cs-2024), HKII (Cell Signaling, cs-2867), GLUT1 (Abcam, ab-40084), GLUT4 (Novus Biologicals, NBP2-22214), Na/K-ATPase (Cell Signaling, cs-3010), UCP1 (Abcam, ab-10983), GAPDH (Cell Signaling, cs-2118), rictor (Cell Signaling, cs-2114).

2-Deoxyglucose uptake. For 2-DG uptake measurement in BAT, mice were housed in single cages at 22°C or 4°C for 4h without food. Subsequently, mice were treated with 32.8ug/kg of 2-DG (Sigma) and sacrificed 45 minutes later. BAT was harvested and lysed in 10mM Tris-HCl (pH 8.1). 2-DG uptake was measured by quantifying 2-DG6P accumulation in BAT using a commercial kit (Cosmo Bio Co, LTD.) following the manufacturer's instructions. Values were normalized to tissue weight.

Hexokinase activity. Crude mitochondrial and cytosolic fractions were isolated from fresh BAT of mice that were housed in single cages at 22°C or 4°C for 4h without food by differential centrifugation. Hexokinase activity of the fractions was determined using a commercial kit (Abcam), following the manufacturer's instructions. Equal amount of protein was used for activity determination.

Histology and Immunostainings. Tissue was fixed over night in 4% paraformaldehyde in PBS at 4°C, dehydrated, embedded in paraffin and cut in 5um thick sections. Sections were stained with H&E (Merck) to perform general histology. For immunostainings the following antibody was used: RFP (ab62341, Abcam). DAPI was used to stain nuclei.

Electron Microscopy. Tissue was fixed in 3% paraformaldehyde/0.5% glutaraldehyde, followed by fixation in 1% osmiumtetroxid and subsequent embedding in epon. Tissue was cut in 60-70nm thick sections and images were taken with a Morgagni 268(D) TEM (FEI).

Blood Analysis. Free fatty acids and glycerol in plasma were determined using a commercial kit (HR Series NEFA-HR(2). Plasma triglycerides were measured using a biochemical analyzer (Cobas c 111 analyzer, Roche).

Recombinant AAV Vector Production and Delivery. AAV vector production and delivery into BAT was carried out as described by Jimenez et al. **(Jimenez et al. 2013)**. Briefly, single-stranded AAV vectors were produced through triple transfection of HEK293 cells and subsequent purification on a CsCl gradient. AAV vectors used were as follows: AAV8-CAG-RFP, AAV8-CAG-humanAkt2^{S474D} and AAV9-CMV-HKII. Noncoding plasmids carrying the CAG or CMV promoter and a multicloning site (AAV8-CAG-null, AAV9-CMV-null) were used to produce null particles. For intra BAT AAV administration, mice were anaesthetized with isoflurane and a longitudinal incision was performed at the interscapular area to expose the BAT. Each lobe of the BAT was injected twice with 10uL of viral solution to distribute the vector in the entire depot. Each mouse received 2x10^{11} viral

genomes dissolved in 0.001% Pluronics in PBS. Mice were allowed to recover from the surgery for 2 weeks before experiments were performed.

Statistical Analysis. All data are expressed as mean ± SEM. To determine statistically significant differences between groups, unpaired Student's t-test was used.

Results

Norepinephrine activates mTORC2 *in vitro* via cAMP, PI3K and Epac1. To investigate the role of mTORC2 signaling in NST, we first examined whether mTORC2 is activated by signals that induce thermogenesis. In particular, differentiated brown adipocytes (dBACs) were treated with NE to induce β-adrenergic signaling. As expected, NE treatment resulted in stimulation of PKA signaling as suggested by increased Creb-S133, HSL-S563, and perilipin-S497 phosphorylation **(Figure 1A)**. Importantly, NE also stimulated phosphorylation of the mTORC2 target sites Akt-S473, -T450, and PKCα-S638/641 **(Figure 1A)**. We note that PKCα-S638/641 phosphorylation results in reduced turnover and thereby elevated levels of total PKCα **(Ikenoue et al. 2008)**. These observations suggest that β-adrenergic stimulation induces mTORC2 signaling, in addition to PKA, in dBACs.

Next, we investigated the pathway via which NE stimulates mTORC2. Insulin activates mTORC2 in a PI3K-dependent but mTORC1-independent manner. We examined whether NE activates mTORC2 in a similar manner. We stimulated dBACs with NE in the presence of the pan-mTOR (mTORC1 and mTORC2) inhibitor Torin, the mTORC1-specific inhibitor rapamycin or the PI3K inhibitor Wortmannin. Similar to insulin-induced mTORC2 stimulation, NE-induced activation of mTORC2 was independent of mTORC1, since pretreatment of dBACs with rapamycin did not prevent induction of Akt-S473 phosphorylation upon NE stimulation **(Figure 1B)**. In contrast, inhibition of mTOR with Torin or of PI3K with Wortmannin prevented Akt-S473 phosphorylation **(Figure 1B)**. Hence, NE-induced activation of mTORC2 in dBACs is dependent on PI3K and independent on mTORC1.

NE stimulation leads to an increase in intracellular cAMP, which is crucial for NE-induced activation of PKA signaling **(Cannon and Nedergaard 2004)**. To test whether cAMP is required for NE-induced activation of mTORC2, we treated dBACs with the cell-permeable cAMP analogue 8-Br-cAMP. Similar to NE stimulation, 8-Br-cAMP treatment induced Akt-S473 phosphorylation in dBACs **(Figure 1C)**. 8-Br-cAMP stimulated mTORC2 signaling when mTORC1 was blocked with rapamycin, but was no longer able to induce Akt-S473 phosphorylation when mTOR or PI3K were inhibited with Torin or Wortmannin, respectively **(Figure 1C)**. Thus, NE induces mTORC2 signaling via cAMP and PI3K.

cAMP has several target proteins, two of which are PKA and Epac1. Epac1 mediates cAMP-induced activation of mTORC2 in prostate cancer cells **(Misra and Pizzo 2012)**, and thus might be involved in cAMP-induced stimulation of mTORC2 in BAT. To investigate whether PKA or Epac1 is required for cAMP-induced activation of mTORC2, we stimulated dBACs with 8-Br-cAMP in the presence of the PKA inhibitor H89 or the Epac inhibitor ESI-09. Interestingly, treatment of dBACs with H89 resulted in a hyper-phosphorylation of Akt-S473, suggesting that inhibition of PKA signaling does not impair activation of mTORC2 **(Figure 1D)**. In contrast, treatment with the Epac inhibitor ESI-09 prevented 8-Br-cAMP induced phosphorylation of Akt-S473, suggesting that NE activates mTORC2 via cAMP, Epac1 and PI3K **(Figure 1D)**.

Norepinephrine and cold activate mTORC2 *in vivo*. We next assessed whether NE stimulation can induce mTORC2 signaling in BAT *in vivo*. In line with our *in vitro* results, treatment of mice with NE induced phosphorylation of the mTORC2 target Akt **(Figure 2A)**. Importantly, AdRiKO mice **(Cybulski et al. 2009)**, which are defective for mTORC2 signaling in both BAT and white adipose tissue (WAT) **(Supplementary Figure 1A)**, did not display induction of Akt-S473 phosphorylation in BAT upon NE stimulation **(Figure 2B)**. Hence, functional mTORC2 is required for Akt phosphorylation in BAT in response to NE. Since NE is released from the brain upon cold exposure, we hypothesized that mTORC2 signaling in BAT could also be induced by cold stress. Similar to the results obtained with NE stimulation, cold exposure

induced Akt-S473 phosphorylation in BAT of control mice **(Figure 2C)**. Again, this induction was dependent on functional mTORC2 signaling as Akt phosphorylation and phosphorylation of the Akt target FoxO1 were not induced in BAT upon cold exposure of AdRiKO mice **(Figure 2C)**. In contrast to BAT, mTORC2 signaling was not induced in subcutaneous WAT (sWAT) upon cold exposure **(Supplementary Figure 1B)**. Taken together, these data demonstrate that mTORC2 signaling is induced by NE and cold in BAT but not in sWAT.

As we observed an induction of mTORC2 signaling in BAT upon NE and cold stimulation, we next investigated whether a defect in mTORC2 signaling affected temperature regulation. AdRiKO mice were hypothermic when housed at 22°C, which is a mild temperature stress for mice (Figure 2D). The hypothermia could not be accounted for by a reduction in locomotor activity **(Supplementary Figure 1C)**. In contrast, housing AdRiKO mice at thermoneutrality (30°C) for 10h, partially prevented this hypothermic phenotype **(Figure 2E)**. Next, we performed an acute cold exposure with AdRiKO and control mice. In contrast to control mice, AdRiKO mice were unable to maintain stable body temperature when housed at 4°C **(Figure 2F)**. Thus, inactivation of mTORC2 signaling in adipose tissue leads to decreased body temperature and increased sensitivity to cold stress.

mTORC2 in adipose tissue is not required for cold-induced lipid droplet mobilization, mitochondrial uncoupling, and β-oxidation. Thermogenesis upon β-adrenergic stimulation requires mobilization of lipid stores, induction of β-oxidation, and stimulation of mitochondrial uncoupling to generate heat. Since AdRiKO mice are hypothermic and exhibit increased sensitivity to cold (see above), we investigated whether AdRiKO mice are defective in lipid mobilization, β-oxidation or mitochondrial uncoupling in adipose tissue. There was no discernible difference in the morphology of lipid droplets in sWAT from AdRiKO mice compared to wild type control mice kept at 22°C. Furthermore, both were able to mobilize sWAT lipid stores upon cold exposure (4°C) as suggested by a reduction in the size of lipid droplets, i.e., the appearance of multilocular adipocytes, in sWAT **(Figure 3A)**. In line with this, cold-exposed

AdRiKO and control mice also displayed a comparable increase in levels of circulating non-esterified fatty acids (NEFAs), i.e., free fatty acids, and glycerol **(Figure 3B and 3C)**. In BAT, however, AdRiKO mice housed at 22°C displayed larger lipid droplets compared to control mice **(Figure 3D)**. At 4°C, lipid droplet size in BAT decreased to the same extent in AdRiKO and control mice **(Figure 3D)**, and free fatty acid levels were even enhanced in the AdRiKO BAT **(Figure 3E)**. These findings suggest that the defect in temperature regulation in AdRiKO mice is most likely not due to impaired release of free fatty acids in sWAT and BAT upon cold exposure.

As mentioned above, cold-exposed AdRiKO mice display significantly increased levels of free fatty acids in BAT compared to control mice **(Figure 3E)**. We hypothesized that this increase in NEFAs might be due to impaired mitochondrial function, which could lead to accumulation of NEFAs in BAT. To test this possibility, we first measured induction of genes involved in mitochondrial uncoupling in BAT upon cold exposure. Despite the cold-sensitive phenotype of AdRiKO mice, mRNA levels of *UCP1*, *Dio2* and *PGC-1*α were induced to a similar extent in BAT of AdRiKO and control mice **(Figure 4A)**, and UCP1 protein levels were also similar **(Figure 4B)**. Second, AdRiKO mice did not exhibit any defect in expression of genes involved in β-oxidation **(Figure 4C)**. Thus, AdRiKO mice appear normal for induction of the thermogenic transcriptional program and expression of β-oxidation genes. Third, we measured expression of mitochondrial proteins in BAT, in particular proteins of the electron transport chain - the so-called mitoprofile. AdRiKO mice displayed a slight decrease (22°C) or no change (4°C) in their mitoprofile compared to control mice **(Figure 4D)**. Fourth, mitochondrial DNA (mtDNA) copy number was unchanged in BAT of AdRiKO mice **(Figure 4E)**, suggesting that BAT of AdRiKO and control mice contain a similar amount of mitochondria. Fifth, EM micrographs of BAT revealed no difference between AdRiKO and control mitochondria with regard to size, shape and cristae structure **(Figure 4F)**. Finally, cold-exposed AdRiKO mice exhibited normal induction of oxygen consumption in BAT **(Figure 4G)** and at the whole body level **(Figure 4H)**. Thus, BAT in AdRiKO mice has normal mitochondrial

function, suggesting that the observed cold sensitivity of AdRiKO mice does not stem from a mitochondrial defect. ,

Despite a similar maximal induction of whole-body respiration **(Figure 4H)**, AdRiKO mice were unable to maintain an enhanced metabolic rate throughout the duration of the cold exposure time course **(Figure 4I)**. This inability to maintain an enhanced metabolic rate may account for the inability of AdRiKO mice to sustain an NST response.

mTORC2 in adipose tissue is required for cold-induced glucose uptake and glycolysis. Glucose uptake and glycolysis are strongly enhanced in BAT upon cold exposure, to compensate for the loss of mitochondrial ATP production due to heat-generating mitochondrial uncoupling **(Greco-Perotto et al. 1987, Vallerand et al. 1990, Hao et al. 2015)**. mTORC2 is an important regulator of insulin-induced glucose uptake and glycolysis in WAT, muscle and liver **(Kumar et al. 2008, Kumar et al. 2010, Hagiwara et al. 2012)**. Thus, reduced glucose uptake and glycolysis might explain the failure of AdRiKO mice to maintain an enhanced metabolic rate upon cold exposure **(Figure 4I)**. To test this notion, we examined cold-induced glucose uptake in BAT. More specifically, we measured 2-deoxyglucose-6-phosphate (2DG6P) accumulation in BAT 45 minutes after injecting mice with 2-deoxyglucose (2DG). BAT in AdRiKO mice displayed significantly impaired glucose uptake upon cold exposure **(Figure 5A)**. Similarly, cold exposed AdRiKO mice failed to increase glycolysis in BAT, as indicated by a reduced extracellular acidification rate (ECAR) by BAT explants **(Figure 5B)**. Thus, mTORC2 signaling is required to induce glucose uptake and glycolysis in BAT upon cold exposure. To determine whether absence of mTORC2 in BAT leads to energetic stress upon cold exposure, which could account for an inability to sustain NST, we examined AMP-activated protein kinase (AMPK) signaling. AMPK is activated in response to low energy levels **(Hardie and Hawley 2001)**. Indeed, AdRiKO mice displayed enhanced phosphorylation of AMPK-T172 and phosphorylation of the AMPK targets ACC and raptor **(Figure 5C)**, indicating energy stress in BAT of these mice.

Next, we investigated how mTORC2 signaling affects glucose uptake and glycolysis in BAT. mTORC2 signaling has been shown to mediate

insulin-stimulated translocation of GLUT4 to the plasma membrane **(Kumar et al. 2010)**. Moreover, GLUT1 is involved in glucose uptake into brown adipocytes upon β-adrenergic stimulation **(Dallner et al. 2006)**. To test whether mTORC2 signaling in BAT affects plasma membrane localization of GLUT1 or GLUT4, we isolated plasma membrane from AdRiKO and control BAT. The amount of GLUT1 and GLUT4 in the two plasma membrane fractions was similar **(Figure 5D)**. This suggests that mTORC2 in BAT does not mediate cold-induced glucose uptake and glycolysis by affecting localization of glucose transporters. Glucose uptake is also affected by hexokinases, which phosphorylate glucose to catalyze the first and rate-limiting step of glycolysis. From the four different hexokinase isoforms, hexokinase I (HKI) and hexokinase II (HKII) are the two dominant isoforms in BAT, and are found both in the cytosol and at mitochondria **(Shinohara et al. 1998, Wilson 2003)**. Immunoblot analysis of cytosolic **(Figure 5E)** and mitochondrial **(Figure 5F)** fractions from BAT of AdRiKO and control mice revealed no significant difference in the amount and subcellular localization of HKI and HKII. However, while mitochondrial hexokinase activity was similar in AdRiKO and control mice, cytosolic hexokinase activity was induced in BAT of cold-exposed control but not AdRiKO mice **(Figure 5G and 5H)**. Thus, mTORC2 signaling in BAT stimulates glucose uptake and glycolysis upon cold exposure via regulation of cytosolic hexokinase activity. Collectively, the above findings suggest that impaired glucose metabolism in BAT of AdRiKO mice accounts for the failure to sustain NST.

Restoration of glucose uptake or Akt signaling suppresses the thermogenic defect in AdRiKO mice. Our data suggest that AdRiKO mice are hypothermic and sensitive to cold exposure due to impaired activation of glucose metabolism in BAT. We therefore reasoned that restoring glucose uptake in BAT could be sufficient to improve temperature regulation. To test this notion, we overexpressed HKII in BAT of AdRiKO and control mice via intra-BAT injection of an adeno-associated virus (AAV) expressing HKII. This technique was used previously to activate glucose uptake specifically in BAT **(Jimenez et al. 2013)**. To confirm that the transgene was specifically

targeted to BAT, we injected an RFP-expressing AAV into the BAT of control mice and measured RFP expression. RFP was strongly expressed in BAT, slightly expressed in liver, and not detected in other tissues **(Supplementary Figure 2A and 2B)**. Intra-BAT injection of an HKII-expressing AAV resulted in a strong increase in HKII mRNA levels in BAT of both AdRiKO and control mice **(Figure 6A)**. Overexpression of HKII enhanced cold-induced glucose uptake in BAT of both AdRiKO and control mice **(Figure 6B)**. Importantly, restoration of glucose uptake suppressed the hypothermic phenotype **(Figure 6C)** and improved cold tolerance **(Figure 6D)** in AdRiKO mice. Thus, restoration of glucose metabolism in BAT of AdRiKO mice is sufficient to reverse the hypothermic and cold sensitive phenotype caused by inactivation of mTORC2.

Next we investigated how mTORC2 regulates glucose metabolism and temperature homeostasis in BAT. Akt is a major downstream effector of mTORC2 and stimulates glucose uptake in skeletal muscle and liver downstream **(Kumar et al. 2008, Hagiwara et al. 2012)**. Furthermore, Akt signaling is activated in BAT upon cold exposure **(Figure 2C)**. Thus, we investigated whether expression of constitutively active Akt2 ($Akt2^{S474D}$) **(Hagiwara et al. 2012)** in BAT of AdRiKO mice could restore glucose uptake and temperature regulation. To this end, we injected AAV expressing $Akt2^{S474D}$ into BAT of AdRiKO and control mice. This resulted in a strong expression of $Akt2^{S474D}$ in BAT of AdRiKO and control mice, while PKCα and PKCα-p638/641 levels were unchanged **(Figure 6E)**. Introduction of $Akt2^{S474D}$ increased body temperature and improved cold tolerance in AdRiKO mice **(Figure 6F and 6G)**. Thus, restoration of Akt activity in BAT of AdRiKO mice improved temperature homeostasis. To investigate whether restoration of Akt activity also suppressed the observed defects in glucose metabolism, we measured cold-induced glucose uptake in BAT of AdRiKO and control mice. Interestingly, cold-induced glucose uptake was restored to control levels in BAT of AdRiKO mice expressing $Akt2^{S474D}$ **(Figure 6H)**. In conclusion, these findings suggest that mTORC2-Akt signaling regulates BAT glucose metabolism and thereby NST.

Discussion

We investigated the role of mTORC2 signaling in the regulation of thermogenesis and cold-induced glucose uptake. We show that mTORC2 signaling is activated in brown adipocytes *in vitro* and *in vivo* upon NE-stimulation and cold exposure via cAMP and Epac1, independently of PKA signaling. We also demonstrate that mTORC2-Akt signaling in BAT mediates cold-induced glucose uptake and glycolysis, and is thereby sustains NST.

We found that mice with inactive mTORC2 signaling in adipose tissue (AdRiKO mice) fail to maintain a metabolic rate required to sustain NST and are thus hypothermic and sensitive to cold stress. This impaired NST response of AdRiKO mice is most likely due to impaired glucose uptake and glycolysis in BAT, which results in the inability to maintain energy homeostasis under cold stress. Importantly, overexpressing HKII restored glucose uptake and glycolysis in BAT, and thereby restored body temperature and improved cold tolerance. These results reveal the importance of glucose metabolism, and its regulation by mTORC2, in the maintenance of NST. Interestingly, Olsen et al. reported that β-adrenergic stimulation induced glucose uptake in brown adipocytes *in vitro* in an mTORC2-dependent manner **(Olsen et al. 2014)**. Furthermore, the authors reported that mTORC2 promotes glucose uptake by stimulating GLUT1 translocation to the plasma membrane in an Akt-independent fashion **(Olsen et al. 2014)**. In contrast to these *in vitro* results, we did not observe a change in GLUT1 plasma membrane localization in BAT of AdRiKO mice. Moreover, our *in vivo* results show that mTORC2 mediates cold-stimulated glucose uptake and glycolysis in an Akt-dependent manner. AdRiKO mice displayed a strong decrease in Akt S473 phosphorylation in BAT and overexpression of a constitutively active Akt2 mutant ($Akt2^{S474D}$) restored glucose uptake, body temperature and increased cold tolerance in AdRiKO mice. The mechanism by which mTORC2 regulates glucose uptake might be different *in vivo* and *in vitro*.

How does mTORC2-Akt signaling in BAT stimulate glucose uptake and glycolysis? We found that AdRiKO mice are defective for induction of cytosolic hexokinase activity in BAT upon cold exposure, whereas mitochondrial hexokinase activity was unaffected. Interestingly, it has been

proposed that mitochondria-associated hexokinase utilizes ATP generated by the mitochondria for glucose phosphorylation **(Wilson 2003)**. However, upon cold stress, mitochondrial ATP production is strongly reduced due to activation of the uncoupling protein UCP1 **(Lindberg et al. 1967, De Meis et al. 2012)**. Thus, in the context of thermogenesis, cytosolic rather than mitochondrial hexokinase may account for the increase in glucose uptake and glycolytic rate. In summary, our data suggest that mTORC2 in BAT specifically activates cytosolic hexokinase, which is in turn required for cold-induced glucose uptake and glycolysis and maintenance of energy homeostasis upon cold stress. Future studies should address the mechanism by which mTORC2-Akt signaling activates cytosolic hexokinase.

We observed that mTORC2 signaling is activated in brown adipocytes upon β-adrenergic stimulation. Similar to insulin-induced activation of mTORC2, we found that β-adrenergic stimulation activates mTORC2 in a PI3K-dependent and mTORC1-independent fashion. Additionally, we found that β-adrenergic signaling stimulates mTORC2 via cAMP and Epac1, independently of PKA signaling. Interestingly, it has been shown previously that mTORC2 signaling is activated in prostate cancer cells in an Epac1-dependent fashion upon cAMP-stimulation **(Misra and Pizzo 2012)**. Thus, induction of mTORC2 signaling upon β-adrenergic stimulation seems to occur in several distinct cell types and could thus represent another major input for mTORC2 activation in addition to growth factors. Our results also suggest that mTORC2-Akt signaling in addition to PKA signaling plays an important role in the NST response.

Loss of mTORC2 impaired temperature homeostasis, but without affecting cold-induced β-oxidation, lipid mobilization and mitochondrial uncoupling. This is in contrast to the study of Hung et al., which found that Myf5 muscle- and BAT progenitor cell-specific *rictor* KO (Myf5-*rictor* KO) mice display increased oxidative metabolism and uncoupling in BAT **(Hung et al. 2014)**. A possible explanation for these seemingly discrepant results could be that Hung et al. used a Myf5-driven Cre recombinase to delete *rictor* at the preadipocyte stage, whereas we used an aP2-driven Cre recombinase to delete *rictor* only in mature adipocytes. A defect in mTORC2 signaling during

adipogenesis could affect mature BAT function and potentially result in changes in oxidative metabolism. Nevertheless, our results demonstrate that inactivation of mTORC2 signaling in mature adipocytes does not affect lipid mobilization, mitochondrial function or oxidative metabolism in BAT.

Taken together, our results demonstrate a novel role for mTORC2 in BAT in the regulation of energy homeostasis and thermogenesis, through Akt-mediated stimulation of glucose uptake and glycolysis. NST and subsequent energy dissipation has been proposed as a novel strategy to treat obesity and decrease the risk of obesity-associated diseases **(Clapham and Arch 2011)**. Additionally, cold-stimulated glucose uptake could be used to normalize blood glucose levels in insulin resistant diabetic patients. Our data suggest that activation mTORC2 in BAT, to stimulate glucose metabolism, could have synergistic effects with NST activators in the treatment of obesity.

Acknowledgements

We would like to thank Professor Brian Hemmings (Basel), Professor Johannes Klein (Lübeck), Professor Stefan Krähenbühl (Basel), and the Imaging Core Facility of the Biozentrum for reagents, equipment and technical support. We acknowledge support from the Swiss National Science Foundation, the Louis Jeantet Foundation, the Werner Siemens Foundation (VA), and the Canton of Basel.

Figure 1

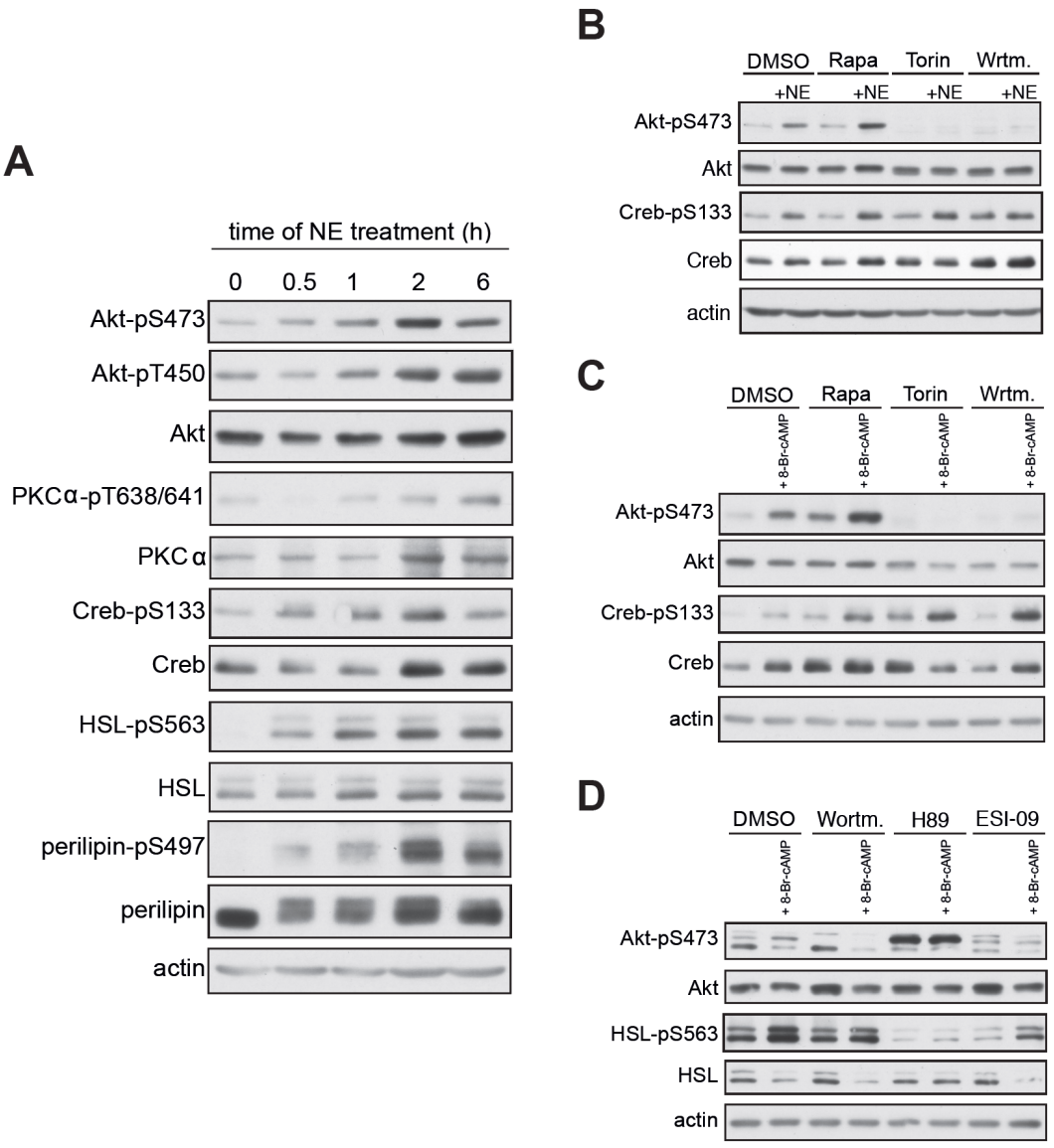


Figure 1. *NE activates mTORC2 in vitro via cAMP, PI3K and Epac1.* (A) Immunoblot analysis of BAT cells stimulated with norepinephrine (NE) for the indicated proteins. (B) Immunoblot analysis of BAT cells stimulated with NE in the presence of Rapamycin (Rapa), Torin or Wortmannin (Wrtm) for the indicated proteins. (C) Immunoblot analysis of BAT cells stimulated with 8-Br-cAMP in the presence of Rapa, Torin or Wrtm for the indicated proteins. (D) Immunoblot analysis of BAT cells stimulated with 8-Br-cAMP in the presence of Wrtm, H89 or ESI-09 for the indicated proteins.

Figure 2

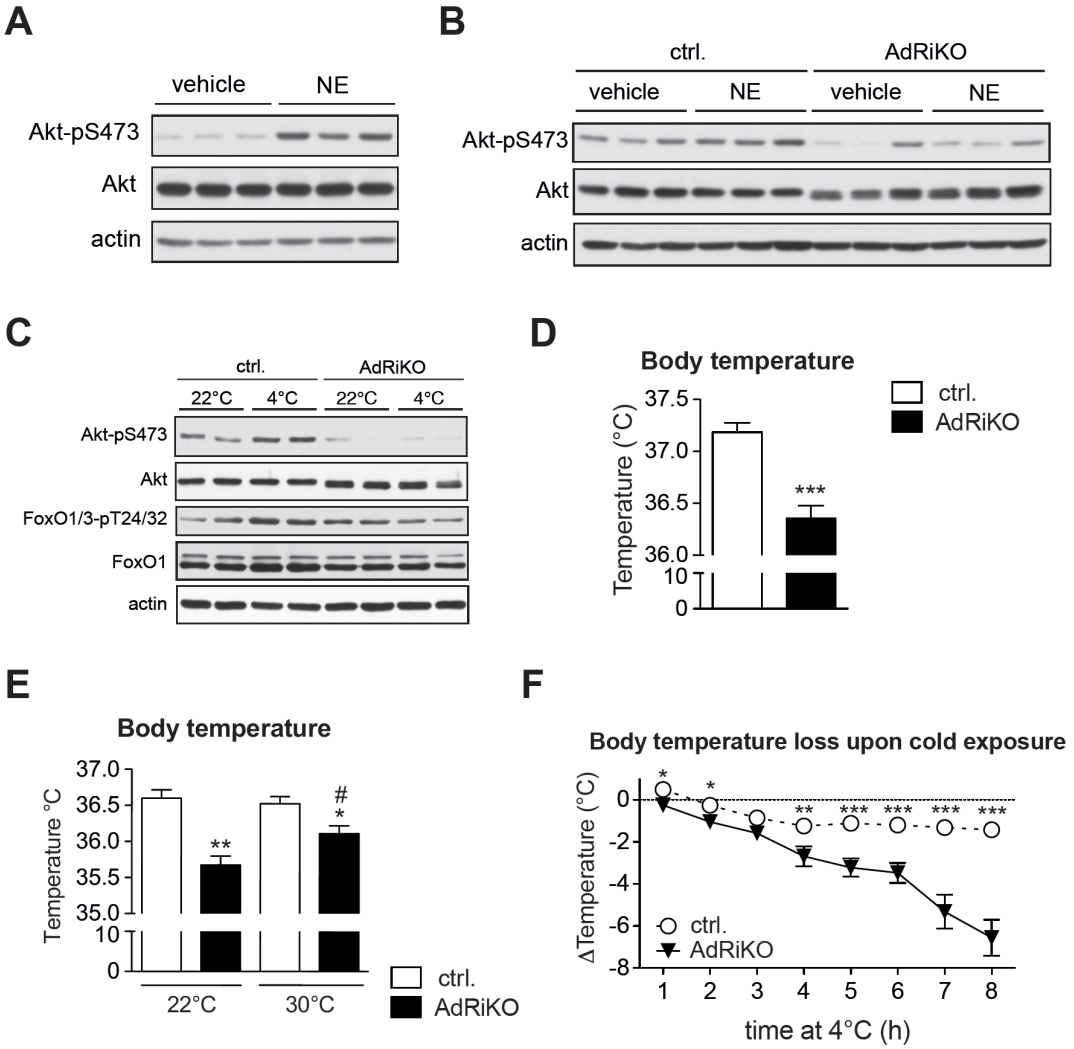


Figure 2. *NE and cold activate mTORC2 in vivo.* (A) Immunoblot analysis of BAT from control mice treated with either norepinephrine (NE) or vehicle for the indicated proteins. (B) Immunoblot analysis of BAT from AdRiKO and control mice treated with either norepinephrine (NE) or vehicle for the indicated proteins. (C) Immunoblot analysis of BAT from AdRiKO and control mice housed at either 22°C or 4°C for the indicated proteins. (D) Body temperature of AdRiKO and control mice housed at 22°C. (E) Body temperature of AdRiKO and control mice housed at 22°C or 30°C. (F) Body temperature loss upon cold exposure of AdRiKO and control mice. Data represent mean ± SEM. Statistically significant differences between AdRiKO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001). Statistically significant differences between temperatures are indicated with a number sign (#=p<0.05; ##=p<0.01; ###=p<0.001).

Figure 3

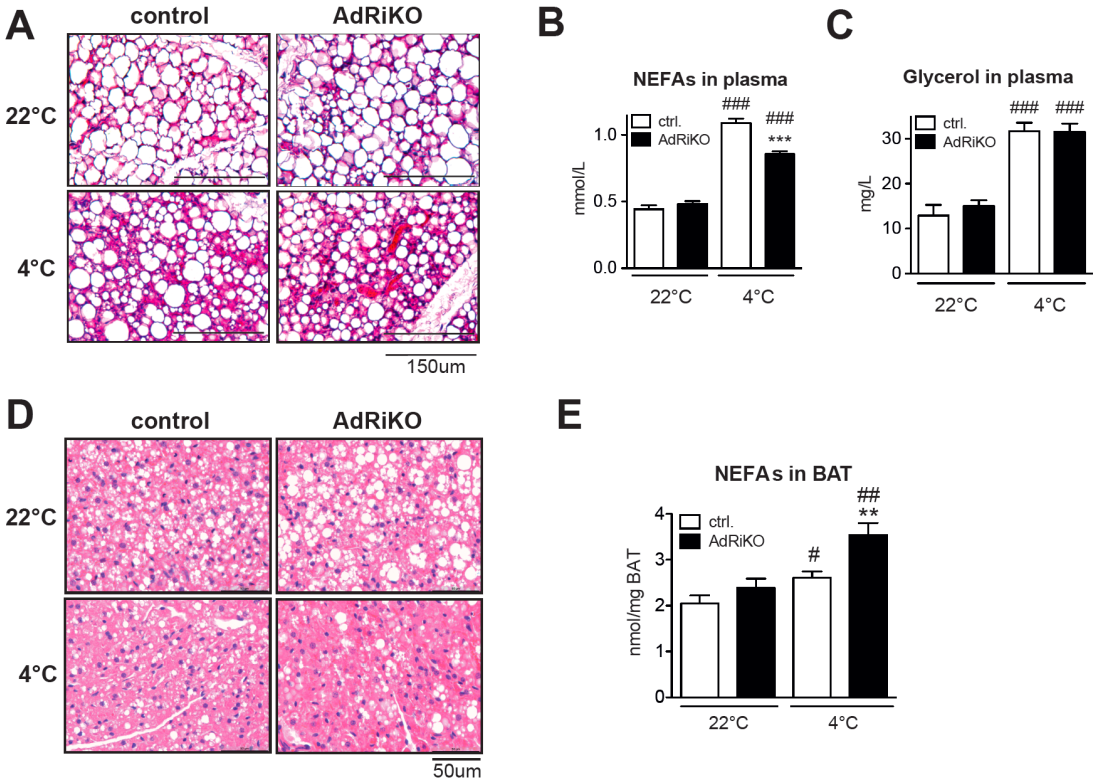


Figure 3. *mTORC2 in adipose tissue is not required for cold-induced lipid droplet mobilization.* (A) H&E staining of sWAT sections from AdRiKO and control mice. (B) Non-esterified fatty acids (NEFAs) in plasma of AdRiKO and control mice. (C) Glycerol in plasma of AdRiKO and control mice. (D) H&E staining of BAT sections from AdRiKO and control mice. (E) NEFAs in BAT of AdRiKO and control mice. Data represent mean ± SEM. Statistically significant differences between AdRiKO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001). Statistically significant differences between temperatures are indicated with a number sign (#=p<0.05; ##=p<0.01; ###=p<0.001).

Figure 4

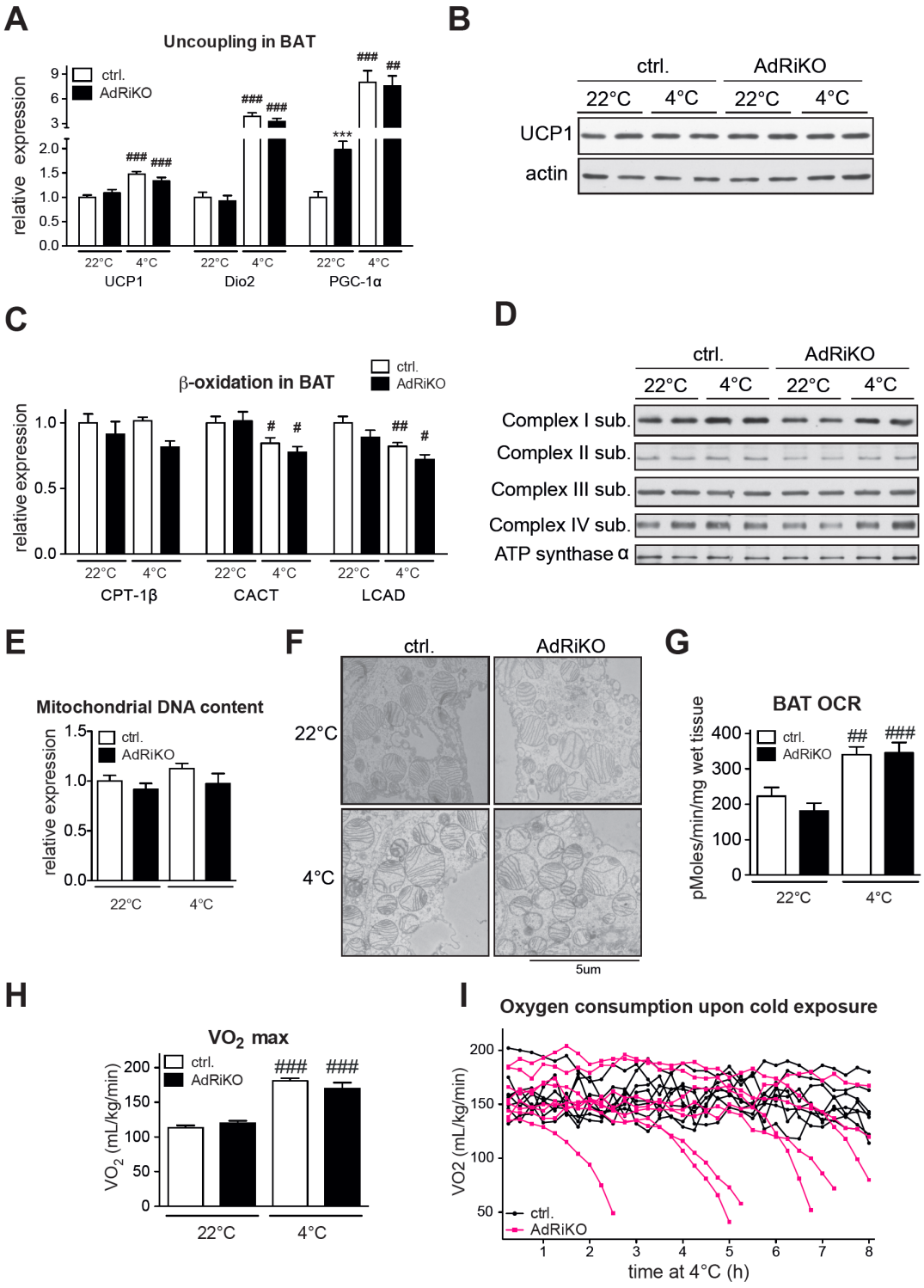

A
Uncoupling in BAT
ctrl.
AdRiKO
relative expression
UCP1
Dio2
PGC-1α
22°C
4°C
B
ctrl.
AdRiKO
UCP1
actin
C
β-oxidation in BAT
CPT-1β
CACT
LCAD
D
Complex I sub.
Complex II sub.
Complex III sub.
Complex IV sub.
ATP synthase α
E
Mitochondrial DNA content
F
5um
G
BAT OCR
pMoles/min/mg wet tissue
H
VO2 max
VO2 (mL/kg/min)
I
Oxygen consumption upon cold exposure
VO2 (mL/kg/min)
time at 4°C (h)

Figure 4. *mTORC2 in adipose tissue is not required for cold-induced mitochondrial uncoupling and β-oxidation*

(A) mRNA levels of the indicated genes in BAT of AdRiKO and control mice. (B) Immunoblot analysis of BAT from AdRiKO and control mice for the indicated proteins. (C) mRNA levels of the indicated genes in BAT of AdRiKO and control mice. (D) Immunoblot analysis of BAT from AdRiKO and control mice for the indicated proteins. (E) Mitochondrial DNA content of BAT from AdRiKO and control mice (F) Electronmicrographs of BAT from AdRiKO and control mice. (G) Oxygen consumption rate (OCR) of BAT explants from AdRiKO and control mice. (H) Maximal respiration (VO_2 max) of AdRiKO and control mice. (I) Respiration (VO_2) of AdRiKO and control mice upon cold exposure. Data represent mean ± SEM. Statistically significant differences between AdRiKO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001). Statistically significant differences between temperatures are indicated with a number sign (#=p<0.05; ##=p<0.01; ###=p<0.001).

Figure 5

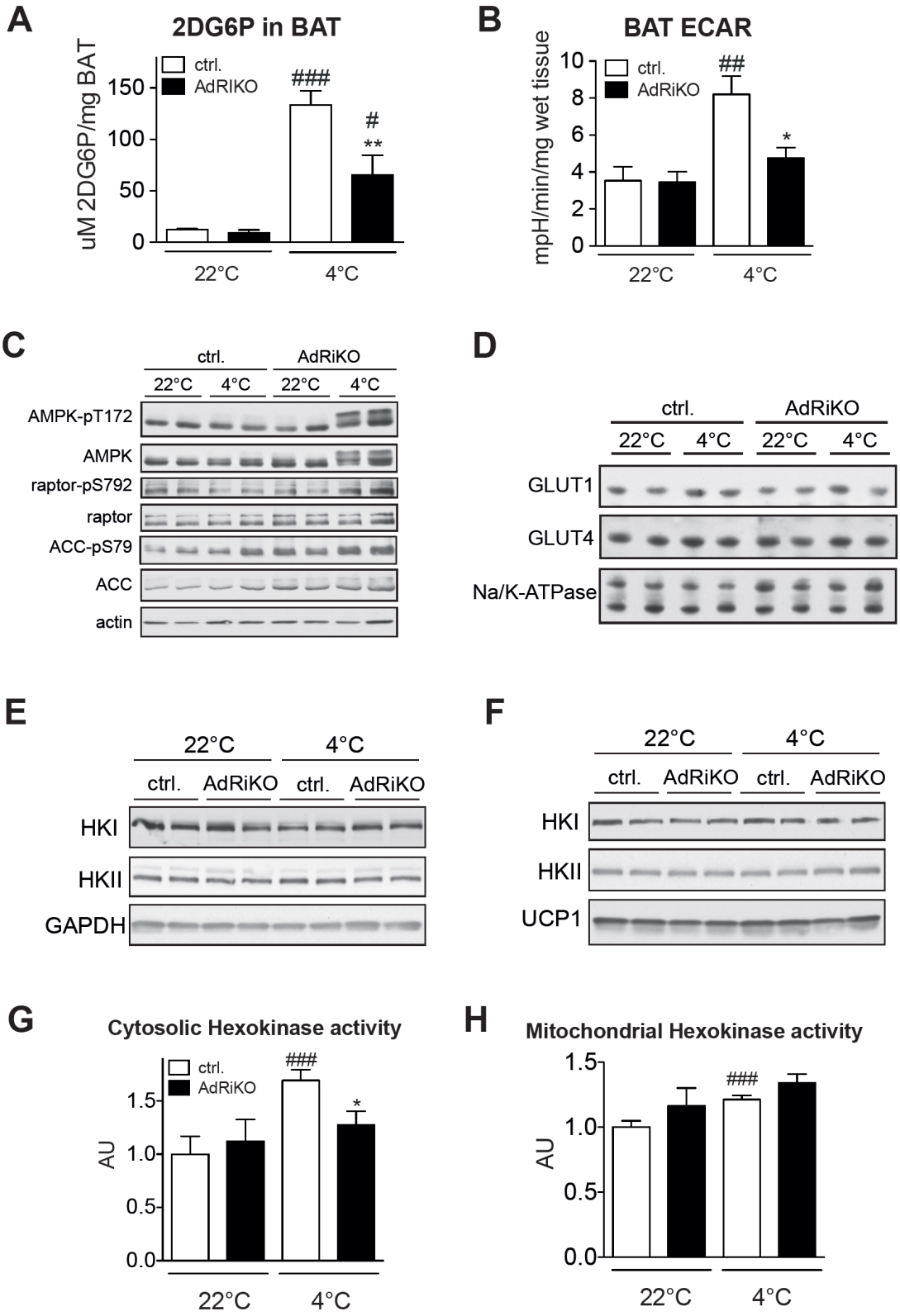

A
2DG6P in BAT
uM 2DG6P/mg BAT
ctrl.
AdRIKO
###
#
**
150
100
50
0
22°C
4°C
B
BAT ECAR
mpH/min/mg wet tissue
ctrl.
AdRiKO
##
*
10
8
6
4
2
0
22°C
4°C
C
ctrl.
AdRiKO
22°C
4°C
22°C
4°C
AMPK-pT172
AMPK
raptor-pS792
raptor
ACC-pS79
ACC
actin
D
ctrl.
AdRiKO
22°C
4°C
22°C
4°C
GLUT1
GLUT4
Na/K-ATPase
E
22°C
4°C
ctrl.
AdRiKO
ctrl.
AdRiKO
HKI
HKII
GAPDH
F
22°C
4°C
ctrl.
AdRiKO
ctrl.
AdRiKO
HKI
HKII
UCP1
G
Cytosolic Hexokinase activity
ctrl.
AdRiKO
###
*
AU
1.5
1.0
0.5
0.0
22°C
4°C
H
Mitochondrial Hexokinase activity
###
AU
1.5
1.0
0.5
0.0
22°C
4°C

Figure 5. *mTORC2 in adipose tissue is required for cold-induced glucose uptake and glycolysis* (A) 2-Deoxyglucose-6-phosphate (2DG6P) accumulation in BAT of AdRiKO and control mice. (B) Extracellular acidification rate (ECAR) of BAT explants from AdRiKO and control mice. (C) Immunoblot analysis of BAT from AdRiKO and control mice for the indicated proteins. (D) Immunoblot analysis of isolated plasma membranes from BAT of AdRiKO and control mice for the indicated proteins. (E) Immunoblot analysis of cytosolic fractions from BAT of AdRiKO and control mice for the indicated proteins. (F) Immunoblot analysis of crude mitochondrial fractions from BAT of AdRiKO and control mice for the indicated proteins. (G) Cytosolic hexokinase activity in BAT of AdRiKO and control mice. (H) Mitochondrial hexokinase activity in BAT of AdRiKO and control mice. Data represent mean ± SEM. Statistically significant differences between AdRiKO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001). Statistically significant differences between temperatures are indicated with a number sign (#=p<0.05; ##=p<0.01; ###=p<0.001).

Figure 6

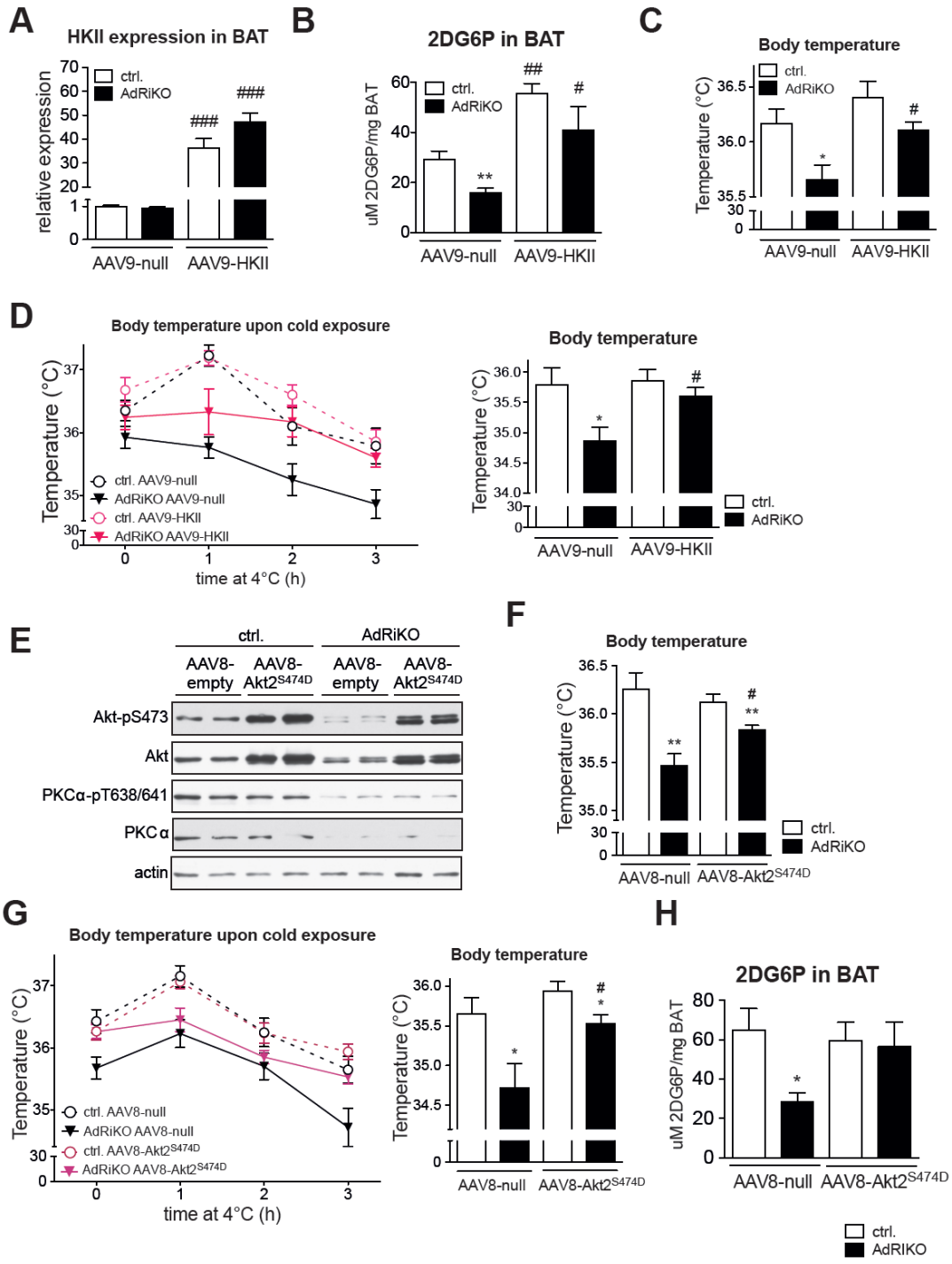
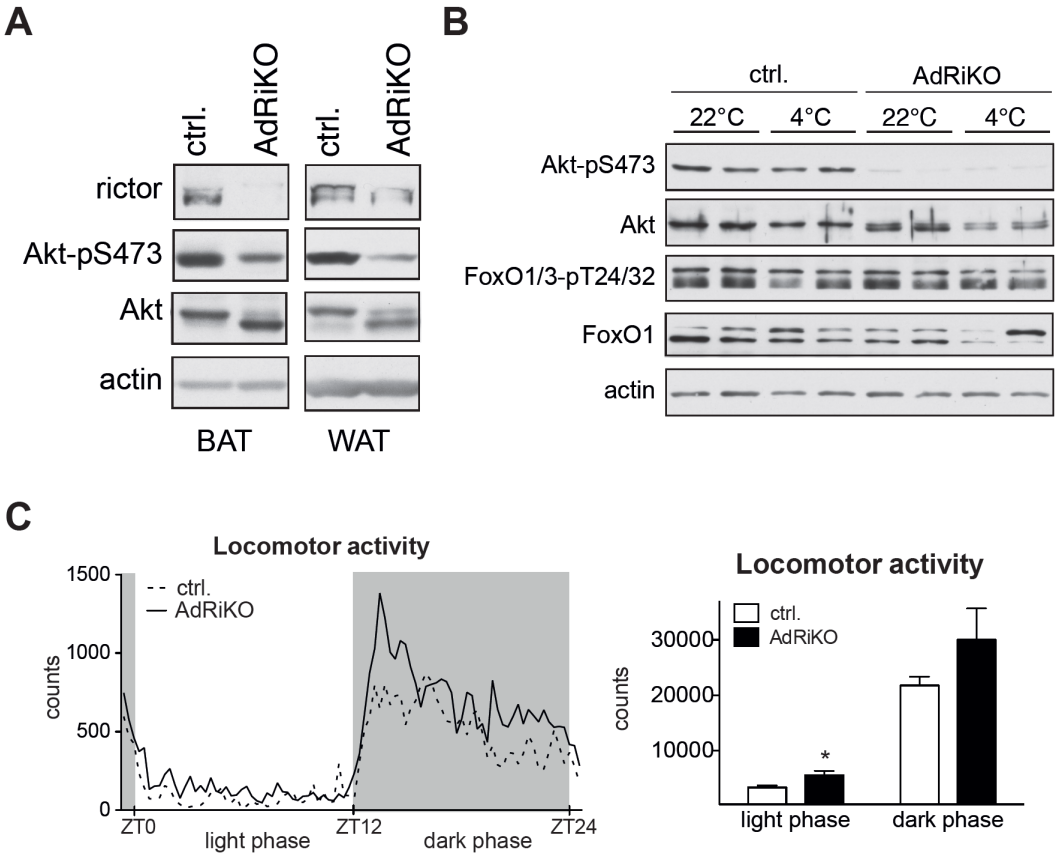

A
HKII expression in BAT
relative expression
ctrl.
AdRiKO
###
###
AAV9-null
AAV9-HKII
B
2DG6P in BAT
uM 2DG6P/mg BAT
ctrl.
AdRiKO
**
##
#
AAV9-null
AAV9-HKII
C
Body temperature
Temperature (°C)
ctrl.
AdRiKO
*
#
AAV9-null
AAV9-HKII
D
Body temperature upon cold exposure
Temperature (°C)
ctrl. AAV9-null
AdRiKO AAV9-null
ctrl. AAV9-HKII
AdRiKO AAV9-HKII
time at 4°C (h)
Body temperature
Temperature (°C)
*
#
ctrl.
AdRiKO
AAV9-null
AAV9-HKII
E
ctrl.
AdRiKO
AAV8-empty
AAV8-Akt2S474D
AAV8-empty
AAV8-Akt2S474D
Akt-pS473
Akt
PKCα-pT638/641
PKCα
actin
F
Body temperature
Temperature (°C)
**
#
**
ctrl.
AdRiKO
AAV8-null
AAV8-Akt2S474D
G
Body temperature upon cold exposure
Temperature (°C)
ctrl. AAV8-null
AdRiKO AAV8-null
ctrl. AAV8-Akt2S474D
AdRiKO AAV8-Akt2S474D
time at 4°C (h)
Body temperature
Temperature (°C)
*
#
*
AAV8-null
AAV8-Akt2S474D
H
2DG6P in BAT
uM 2DG6P/mg BAT
*
AAV8-null
AAV8-Akt2S474D
ctrl.
AdRIKO

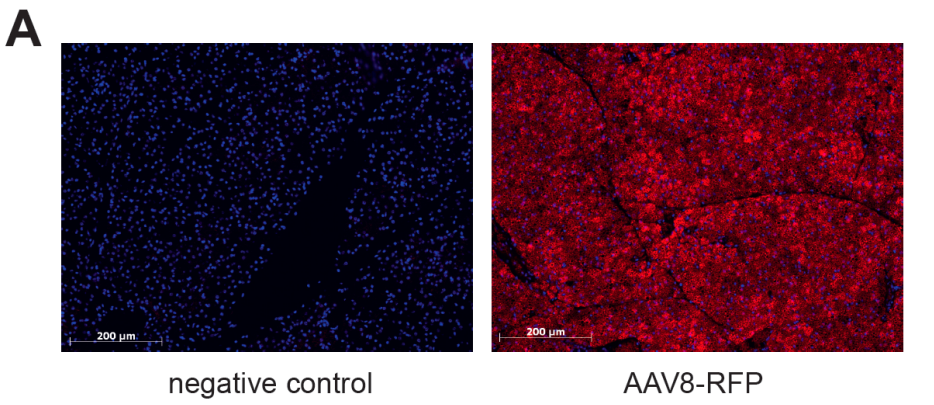
Figure 6. *Restoration of glucose uptake or Akt signaling suppresses the thermogenic defect in AdRiKO mice.* (A) HKII mRNA expression level in BAT of AdRiKO and control mice infected with either AAV9-HKII or AAV9-empty. (B) Cold-induced 2-Deoxyglucose-6-phosphate (2DG6P) accumulation in BAT of AdRiKO and control mice infected with either AAV9-HKII or AAV9-empty. (C) Body temperature of AdRiKO and control mice infected with either AAV9-HKII or AAV9-empty. (D) Body temperature upon cold exposure of AdRiKO and control mice infected with either AAV9-HKII or AAV9-empty. (E) Immunoblot analysis of BAT from AdRiKO and control mice infected with either AAV8-Akt2^{S474D} or AAV8-empty. (F) Body temperature of AdRiKO and control mice infected with either AAV8-Akt2^{S474D} or AAV8-empty. (G) Body temperature upon cold exposure of AdRiKO and control mice infected with either AAV8-Akt2^{S474D} or AAV8-empty. (H) Cold-induced 2-Deoxyglucose-6-phosphate (2DG6P) accumulation in BAT of AdRiKO and control mice infected with either AAV8-Akt2^{S474D} or AAV8-empty. Data represent mean ± SEM. Statistically significant differences between AdRiKO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001). Statistically significant differences between viruses are indicated with a number sign (#=p<0.05; ##=p<0.01; ###=p<0.001).

Supplementary Figure 1, Related to Figure 2



Supplementary Figure 1, Related to Figure 2. *Cold does not activate mTORC2 in sWAT.* (A) Immunoblot analysis of BAT and sWAT of AdRiKO and control mice for the indicated proteins. (B) Immunoblot analysis of sWAT of AdRiKO and control mice for the indicated proteins. (C) Locomotor activity of AdRiKO and control mice. Data represent mean ± SEM. Statistically significant differences between AdRiKO and control mice are indicated with asterisks (*=p<0.05; **=p<0.01, ***=p<0.001).

Supplementary Figure 2, Related to Figure 6



Supplementary Figure 2, Related to Figure 6. *Intra-BAT injection targets genes of interest specifically to BAT.* (A) Immunostainings for RFP of BAT from control mice infected with either AAV8-RFP or AAV8-empty. (B) RFP mRNA expression in BAT, liver, quadriceps, and WAT of control mice infected with either AAV8-RFP or AAV8-empty. Data represent mean ± SEM.

Supplementary Table 1. Primer sequences used for qRT-PCR

Target	forward primer (5'-3')	reverse primer (5'-3')
CACT	CTGCGCCCATCATTGGA	CAGACCAAACCCAAAGAAGCA
CPT-1β	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
Dio2	GAGGAAGGAAGAAGAGGAAGCAA	TTCTTCCAGTGTTTTGGACA TGC
E-loop	GGTTCTTACTTCAGGGCCATCA	GATTAGACCCGTTACCATCGAGAT
HKII	AAAACCAAGTGCAGAAGGTTGAC	GAACCGCCTAGAAATCTCCAGAA
LCAD	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
Ndufv1	CTTCCCCACTGGCCTCAAG	CCAAAACCCAGTGATCCAGC
PGC-1α	GAGAATGAGGCAAACTTGCTAGCG	TGCATGGTTCTGAGTGCTAAGACC
RFP	GCGGCCACTACACCTGCGAC	TCGGCGTGCTCGTACTGCTC
RPL0	CTGCTGAACATGCTGAACATCTC	CTTCAGGGTTATAAATGCTGCCG
TBP	TGCTGTTGGTGATTGTTGGT	CTGGCTTGTGTGGGAAAGAT
UCP1	TGATGAAGTCCAGACAGACAGTG	TTATTCGTGGTCTCCCAGCATAG

Chapter 4: Discussion and outlook

4. Discussion and outlook

The aim of this thesis was to gain a better understanding of how mTOR signaling in different metabolic organs regulates whole body metabolism. To this end, we studied mTORC1 signaling in orexigenic neurons in the hypothalamus and mTORC2 signaling in adipose tissue to understand their role in the regulation of systemic energy homeostasis.

In the first part of this thesis, we studied the effects of mTORC1 inactivation in orexigenic Agrp neurons on feeding behavior and systemic energy metabolism. It has been previously shown that hypothalamic mTORC1 is involved in the regulation of feeding behavior and energy homeostasis. For example, hyper-activation of mTORC1 in POMC neurons results in hyperphagia-induced obesity **(Mori et al. 2009)** and mTORC1 signaling in the ARC is sensitive to the feeding state **(Cota et al. 2006)**. In line with previous findings **(Cota et al. 2006)**, we observed that mTORC1 activity in the ARC was induced by feeding and down-regulated by fasting. Moreover, we found that mTORC1 signaling in the ARC was circadian, displaying the highest intensity at ZT18, which corresponds to the active feeding period of the mice. This finding is in agreement with other studies, showing that mTORC1 signaling activity displays a circadian pattern in several organs **(Cao et al. 2011, Jouffe et al. 2013, Cornu et al. 2014, Khapre et al. 2014)**.

Interestingly, we found that also *Agrp* and *NPY* mRNA expression was circadian, showing the highest expression at ZT18. Since the circadian *Agrp* and *NPY* expression pattern matched the circadian mTORC1 activity, we hypothesized that mTORC1 signaling in Agrp neurons regulates *Agrp* and *NPY* expression. Indeed, Agrp-*raptor* KO mice, which have no mTORC1 activity in Agrp neurons, exhibited no circadian oscillation of *Agrp* and *NPY* expression. Instead, *Agrp* and *NPY* levels were constitutively reduced. These data strongly suggest that mTORC1 signaling in Agrp neurons regulates circadian oscillation of *Agrp* and *NPY* levels either via a direct or an indirect mechanism. However, despite low *Agrp* and *NPY* levels, Agrp-*raptor* KO mice consumed an equal amount of food compared to control mice.

Interestingly, several studies have shown that inhibition of *Agrp* or *NPY* expression does not affect feeding behavior in mice **(Erickson et al. 1996, Marsh et al. 1998, Palmiter et al. 1998, Qian et al. 2002)**. These findings could thus explain why Agrp-*raptor* KO mice display a similar food intake compared to control mice despite low levels of *Agrp* and *NPY*. Moreover, Agrp-*raptor* KO mice were able to induce *Agrp* and *NPY* expression after overnight starvation even though basal *Agrp* and *NPY* levels were low and the circadian oscillation of these orexigenic neuropeptides was absent in these mice. Based on these findings, we suggest that circadian- and starvation-induced expression of orexigenic neuropeptides is regulated by two distinct mechanisms. While the circadian *Agrp* and *NPY* expression is mTORC1-dependent, the starvation-induced expression of orexigenic neuropeptides is mTORC1-independent. Interestingly, this is in line with our finding that mTORC1 signaling is inactive in the ARC after overnight starvation. Hence, mTORC1 signaling is unlikely to mediate the starvation-induced increase in orexigenic neuropeptide expression and Agrp-*raptor* KO mice therefore display a normal induction of orexigenic neuropeptide expression upon starvation.

What are the mechanisms that regulate circadian- and starvation-induced expression of *Agrp* and *NPY*? It has been proposed that insulin inhibits *Agrp* expression via activation of Akt and subsequent nuclear exclusion and inactivation of FoxO1 **(Plum et al. 2006)**. Upon starvation, Akt activity is low, resulting in activation of FoxO1 and subsequent stimulation of *Agrp* expression. Thus, absence of insulin could be a potential mechanism to induce *Agrp* expression in a fasted state. However, in Agrp-*raptor* KO mice, Akt signaling is constantly hyper-activated due to absence of the negative feedback loop from S6K to IRS-1 **(Harrington et al. 2004, Shah et al. 2004, Um et al. 2004, Harrington et al. 2005)**. If the Akt/FoxO1 pathway described above was the main regulator of *Agrp* expression, *Agrp* expression would be constantly repressed in Agrp-*raptor* KO mice. Contrarily, Agrp-*raptor* KO mice displayed a significant induction of *Agrp* expression upon starvation, which suggests that there is an Akt/FoxO1-independent signaling pathway that stimulates *Agrp* expression upon starvation.

It is also unlikely that insulin/Akt/FoxO1 signaling regulates the circadian expression pattern of *Agrp* and *NPY*. In mice, circulating insulin levels are low during the light phase and increase during the dark phase. If the insulin/Akt/FoxO1 pathway were the main regulator of circadian *Agrp* expression, the levels of orexigenic neuropeptides would be low during the dark phase, since insulin represses the action of FoxO1 on *Agrp* transcription **(Matsuzaki et al. 2003)**. In contrast to this, we found that *Agrp* and *NPY* expression was highest during the dark phase. Hence, our observation suggests that the circadian expression pattern of *Agrp* and *NPY* is positively regulated by anabolic signaling. Moreover, since Agrp-*raptor* KO mice failed to show a circadian oscillation of *Agrp* and *NPY* expression, mTORC1 might be a direct regulator of circadian orexigenic neuropeptide expression. Future studies should be aimed at identifying the transcription factors that regulate the circadian- and starvation-induced orexigenic neuropeptide expression. This could for example be achieved through reverse chromatin immunoprecipitaion (ChIP) **(Dejardin and Kingston 2009)**. IP of the *Agrp* or *NPY* promoter regions followed by Mass Spectrometry allows the identification of proteins associated with these promoters. Analysis of the bound proteins at different times of the day or in different feeding states could reveal novel transcription factors that mediate circadian- and fasting-induced orexigenic neuropeptide expression. Interestingly, the Champion ChIP Transcription Factor Search Portal (SABiosciences) predicted binding sites for SREBP-1c and c-Myc within the *Agrp* and *NPY* promoter. These transcription factors are downstream targets of mTORC1 signaling **(West et al. 1998, Duvel et al. 2010)**. Thus, mTORC1 might stimulate orexigenic neuropeptide expression via SREBP-1c and/or c-Myc.

Analysis of the systemic metabolic phenotype of Agrp-*raptor* KO mice revealed that on a normal and on a high fat diet, Agrp-*raptor* KO mice did not display any difference in body weight, metabolic rate and feeding behavior compared to control mice. Hence, this demonstrates that mTORC1 signaling in Agrp neurons is dispensable for the regulation of feeding behavior and energy homeostasis. This observation is in line with a recent study showing that hyper-activation of mTORC1 in Agrp neurons did not cause any metabolic alterations **(Yang et al. 2012)**. In contrast to this, as mentioned

earlier, hyper-activation of mTORC1 in POMC neurons resulted in hyperphagic and obese mice **(Mori et al. 2009)**. Interestingly, a similar observation has been made for mTORC2: While specific deletion of *rictor* in POMC neurons caused abnormal feeding behavior, resulting in obesity; Agrp neuron-specific inactivation of mTORC2 did not lead to any metabolic alterations **(Kocalis et al. 2014)**. These findings suggest that mTORC1 and mTORC2 signaling are important for POMC neuronal function while they are dispensable for Agrp neuronal function. Alternatively, compensatory mechanisms could have developed in the mice with Agrp neuron-specific alterations in mTOR signaling, circumventing the dependence on mTOR in Agrp neurons for the regulation of feeding behavior and energy homeostasis. In line with this hypothesis, ablation of Agrp neurons in neonatal mice does not result in any alterations in feeding behavior and energy metabolism. Contrariwise, deletion of Agrp neurons in adult mice results in a strong decrease in food intake leading to death due to starvation **(Luquet et al. 2005)**. Thus, using an inducible Agrp-Cre line would allow modulation of mTOR signaling in Agrp neurons in adult mice. This would circumvent the development of possible compensatory regulations and could help answer the question whether mTOR signaling affects Agrp neuronal function.

Taken together, characterization of Agrp-*raptor* KO mice revealed that mTORC1 signaling in Agrp neurons regulates circadian but not starvation-induced expression of orexigenic neuropeptides without affecting feeding behavior and systemic energy homeostasis. However, due to the possibility that compensatory mechanisms could have masked the effect of *raptor* deletion on Agrp neuronal function, it cannot be excluded that mTORC1 signaling in Agrp neurons nevertheless could play a role in the regulation of energy homeostasis and feeding behavior.

In the second part of this thesis we investigated the role of adipose mTORC2 signaling in non-shivering thermogenesis (NST). NST-induced energy dissipation and glucose uptake is a promising strategy to induce weight loss in obese subjects and to normalize blood glucose levels in insulin-resistant diabetic patients **(Clapham and Arch 2011)**. Identification of novel

regulators involved in NST and cold-induced glucose uptake could thus help develop new treatment options for patients with metabolic disorders.

It is well established that mTORC2 signaling is crucial for insulin-stimulated glucose uptake and glycolysis in skeletal muscle, liver and WAT **(Kumar et al. 2008, Kumar et al. 2010, Hagiwara et al. 2012)**. Moreover, mTORC2 signaling is also involved in the regulation of lipid metabolism **(Hagiwara et al. 2012)**. We therefore hypothesized that mTORC2 in BAT might be involved in the regulation of NST and/or cold-induced glucose uptake.

Upon cold stress, NST is induced in BAT via NE-mediated stimulation of β-adrenergic receptors. Interestingly, we found that mTORC2 was activated by β-adrenergic stimulation in brown adipocytes *in vitro* and *in vivo*. Similar to insulin-mediated stimulation of mTORC2, we demonstrated that β-adrenergic activation of mTORC2 was dependent on PI3K. Hence, these findings imply that both growth factor- and β-adrenergic signaling converge on PI3K to activate mTORC2. How does β-adrenergic signaling activate PI3K? Our results suggest that the increase in intracellular cAMP upon β-adrenergic stimulation leads to activation of PI3K-mTORC2 signaling, since treatment of dBACs with 8-Br-cAMP stimulated mTORC2 activity. Surprisingly, we found that the cAMP-mediated stimulation of mTORC2 was independent of the cAMP-dependent protein kinase PKA. Instead, we demonstrated that NE activates mTORC2 in brown adipocytes via cAMP-mediated stimulation of Epac1. Interestingly, in line with our findings in brown adipocytes, it has previously been shown that cAMP activates mTORC2 via Epac1 in prostate cancer cells **(Misra and Pizzo 2012)**. Moreover, Epac1 was shown to stimulate mTORC2 by activating PI3K **(Mei et al. 2002)**. Collectively, these and our findings imply that β-adrenergic stimulation activates mTORC2 in several distinct cell types and could thus constitute a novel major input for mTORC2 signaling besides growth factors. In light of this, it would be interesting to identify additional cell types where mTORC2-signaling is activated in response to β-adrenergic stimulation. Moreover, it would also be important to identify the physiological contexts under which such stimulation occurred. For instance, β-adrenergic signaling in skeletal muscle and heart is

involved in the regulation of muscle mass, fiber type and contractility **(Madamanchi 2007, Lynch and Ryall 2008)**. β-adrenergic stimulation-induced activation of mTORC2 could therefore also play an important role for heart and skeletal muscle function.

Interestingly, in analogy with our findings for mTORC2, we observed that mTORC1 signaling was induced with NE and cAMP stimulation in dBACs *in vitro* and with NE and cold in BAT *in vivo* **(Figures 1A-D)**.

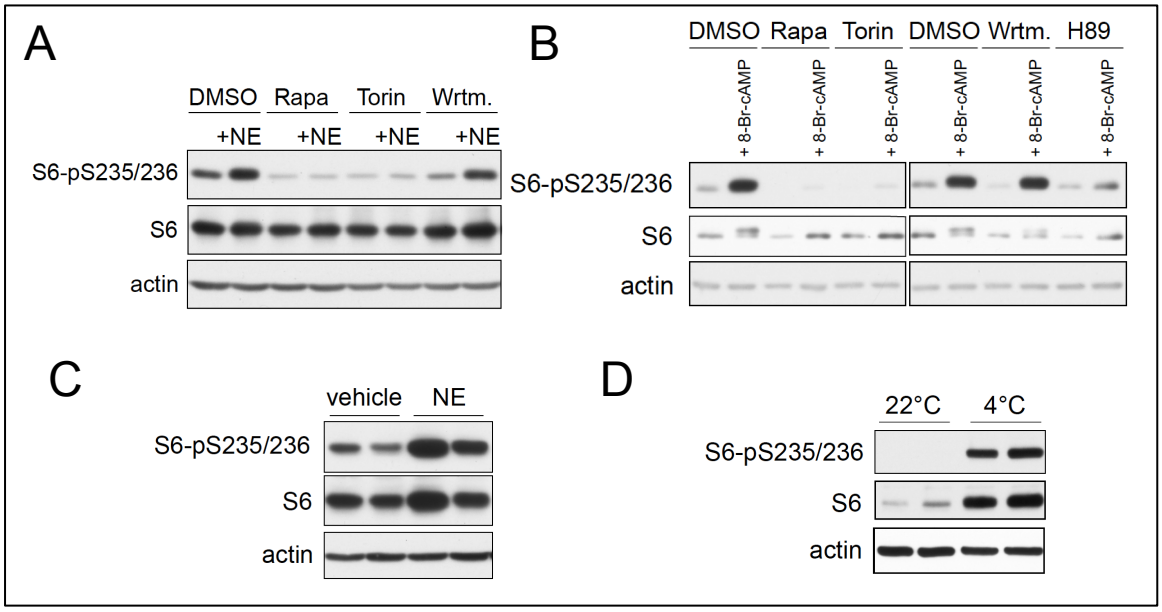


Figure 1. mTORC1 is activated by β-adrenergic signaling in brown adipocytes *in vitro* and *in vivo*. **(A)** Immunoblot analysis of dBACs after NE stimulation in the presence of DMSO, Rapamycin (Rapa), Torin, or Wortmannin (Wrtm.) for the indicated antibodies. **(B)** Immunoblot analysis of dBACs after 8-Br-cAMP stimulation in the presence of DMSO, Rapa, Torin, Wrtm., or H89 for the indicated antibodies. **(C)** Immunoblot analysis of BAT from wild type mice after NE stimulation for the indicated antibodies. **(D)** Immunoblot analysis of BAT from mice that were either housed at 22°C or 4°C for 2h for the indicated antibodies.

While activation of mTORC2 by NE and cAMP was dependent on PI3K, inhibition of PI3K did not prevent NE- and cAMP-induced stimulation of mTORC1 **(Figures 1A and 1B)**. However, mTORC1 activation by cAMP-stimulation was impaired when PKA was inhibited with H89 (Figure 1B), suggesting that NE and cAMP activate mTORC1 in a PKA-dependent but PI3K-independent manner. Interestingly, it was shown in a recent study that mice with hyperactive PKA signaling in adrenal cortex display increased mTORC1 activity in adrenal cortical cells **(de Joussineau et al. 2014)**. This

result corroborates our findings obtained in dBACs that PKA might be a novel upstream regulator of mTORC1. Future studies should be aimed at identifying the molecular mechanism through which PKA signaling affects mTORC1 activity. It is well known that the TSC complex, a negative upstream regulator of mTORC1 signaling, is a crucial signaling hub through which many pathways impinge on mTORC1. For example, AMPK, Erk (MAPK pathway), GSK-3β (Wnt signaling), and Akt (PI3K signaling) phosphorylate TSC2 at various sites to either activate or inhibit the TSC complex, which subsequently affects mTORC1 activity **(Dan et al. 2002, Manning et al. 2002, Potter et al. 2002, Corradetti et al. 2004, Inoki et al. 2006, Ma et al. 2007)**. Thus, PKA signaling could potentially phosphorylate TSC2 and thereby inhibit the TSC complex, which leads to activation of mTORC1. Analysis of TSC2 phosphorylation sites by Mass Spectrometry in dBACs upon cAMP stimulation in the presence and absence of PKA inhibition could for example reveal potential regulatory phosphorylation sites on TSC2 that are affected by PKA signaling upon β-adrenergic stimulation. Taken together, our findings demonstrate that both mTORC1 and mTORC2 are activated upon β-adrenergic stimulation in brown adipocytes, however through different mechanisms **(Figure 2)**.

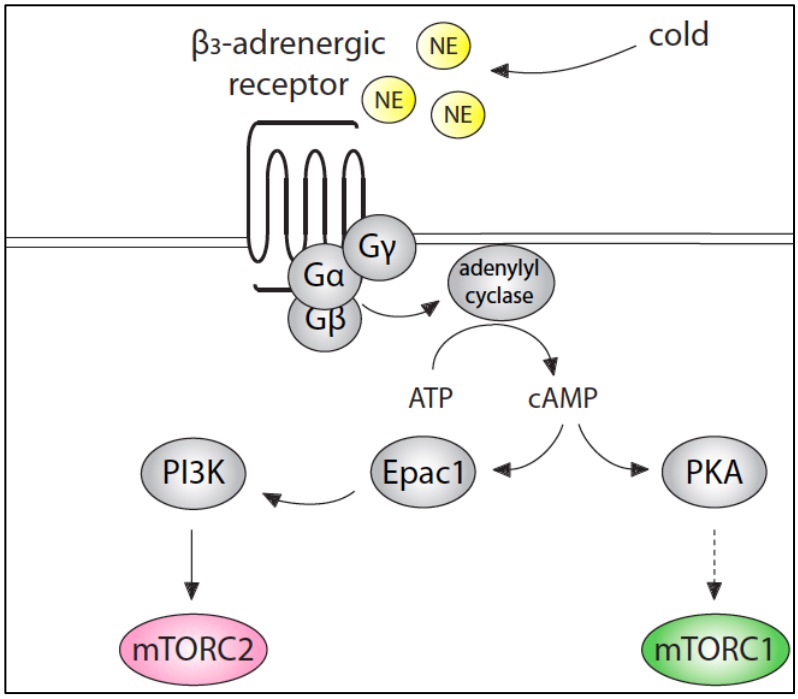


Figure 2. Schematic overview of how β-adrenergic signaling affects mTORC1 and mTORC2 in BAT. For details see text.

Since we observed increased mTORC2 signaling in BAT with cold exposure, we hypothesized that mTORC2 in BAT affects NST. Indeed, we found that AdRiKO mice were hypothermic and sensitive to cold stress, suggesting that mTORC2 signaling in adipose tissue is an important regulator of temperature homeostasis. While AdRiKO mice did not display any defects in the induction of lipid mobilization, oxidative metabolism and mitochondrial uncoupling in BAT upon cold exposure, AdRiKO mice showed a strong impairment in cold-induced glucose uptake and glycolysis. Induction of glucose metabolism in BAT upon cold exposure is a critical process to maintain cellular ATP levels, since mitochondrial ATP production is reduced due to activation of UCP1 and subsequent uncoupling **(Lindberg et al. 1967, De Meis et al. 2012)**. Importantly, restoration of glucose metabolism by overexpression of hexokinase II in BAT of AdRiKO mice was sufficient to restore basal body temperature and improve cold tolerance. Collectively, these data highlight the important role of BAT glucose metabolism in organismal temperature regulation. Moreover, our findings suggest that mTORC2 in BAT regulates temperature homeostasis by stimulating glucose metabolism when mitochondria are uncoupled to maintain energy homeostasis.

Interestingly, a recent study by Olsen et al. found that mTORC2 is required for β-adrenergic-induced glucose uptake in brown adipocytes *in vitro* **(Olsen et al. 2014)**. Olsen et al. proposed that mTORC2 regulates this process by stimulating GLUT1 translocation to the plasma membrane independently of Akt **(Olsen et al. 2014)**. In contrast to this, we could not observe any defect in GLUT1 plasma membrane localization in BAT of AdRiKO mice. Hence, mTORC2 might regulate glucose uptake and glycolysis via different mechanisms *in vivo* and *in vitro*. How does mTORC2 regulate cold-induced glucose uptake and glycolysis *in vivo*? Our data suggest that mTORC2 stimulates glucose uptake and glycolysis in BAT via its downstream target Akt, since over-expression of a constitutively active version of Akt2 ($Akt2^{S474D}$) in BAT of AdRiKO mice restored cold-induced glucose uptake, body temperature and improved cold tolerance in these mice. These data demonstrate that in analogy with insulin-mediated glucose uptake, also β-

adrenergic stimulation-induced glucose uptake is dependent on PI3K-mTORC2-Akt signaling in BAT *in vivo*. It is well established that Akt mediates insulin-induced stimulation of glucose uptake via translocation of GLUT4 to the plasma membrane **(Kumar et al. 2010)**. However, in analogy to our findings regarding GLUT1, we could not observe any defect in GLUT4 plasma membrane localization in BAT of cold-exposed AdRiKO mice. Hence, our data suggests that translocation of glucose transporters to the plasma membrane is not likely to mediate cold-induced glucose uptake via Akt in BAT *in vivo*. Interestingly, we detected an induction of cytosolic hexokinase activity in BAT of control mice upon cold exposure, which could account for the cold-induced increase in glucose uptake and glycolysis in BAT. It has been proposed that mitochondria-associated hexokinase uses ATP generated in the mitochondria to phosphorylate glucose **(Wilson 2003)**. However, upon cold exposure, mitochondrial ATP production is strongly reduced in BAT due to activation of UCP1 **(Lindberg et al. 1967, De Meis et al. 2012)**. Thus, our data suggests that induction of cytosolic hexokinase activity might mediate the increase in glucose uptake and glycolysis with cold exposure in BAT. Interestingly, AdRiKO mice displayed a defect in the induction of cytosolic, but not mitochondrial, hexokinase activity with cold exposure. mTORC2 signaling is thus required for induction of cytosolic hexokinase activity with cold exposure. How does mTORC2 in BAT affect cytosolic hexokinase activity? Hexokinase II is a known target of Akt **(Gottlob et al. 2001, Miyamoto et al. 2008)**. Thus, mTORC2 might regulate cytosolic hexokinase via its downstream target Akt. Interestingly, a recent publication by Betz et al. **(Betz et al. 2013)** demonstrated that insulin stimulation leads to mTORC2-Akt-mediated phosphorylation of hexokinase II at T473, which in turn results in the translocation of hexokinase II from the cytosol to the mitochondria. However, in contrast to Betz et al., we did not observe any changes in subcellular localization of hexokinase II upon cold exposure. Nevertheless, the Akt-mediated phosphorylation of hexokinase II might still regulate induction of cytosolic hexokinase II activity in BAT upon cold exposure. Future experiments should be performed to address this possibility. Furthermore, even though only the hexokinase II isoform is known to contain an Akt phosphorylation motif, also other hexokinase isoforms could potentially be

involved in cold-induced activation of cytosolic hexokinase activity. This possibility could be investigated with immunoprecipitation experiments using an Akt-phospho substrate antibody followed by immunoblotting for different hexokinase isoforms. Moreover, Mass Spectrometry analysis of the immunoprecipitates could furthermore reveal the precise hexokinase phosphorylation site(s) that are potentially phosphorylated by Akt in BAT upon cold exposure. Subsequent generation and analysis of phospho-mimetic or -deficient hexokinase mutants *in vitro* and *in vivo* could then be used to determine the role of these potential Akt phosphorylation sites regarding induction of glucose metabolism upon cold exposure.

As mentioned earlier, we also found that mTORC1 is activated upon NE stimulation and cold exposure in BAT *in vivo* **(Figures 1C and 1D)**. Thus, it would be interesting to investigate in future studies the significance of mTORC1 induction in BAT upon cold stress for NST. Interestingly, adipose tissue specific *raptor* KO (AdRaKO) mice display a browning phenotype of WAT, while UCP1 expression is unaltered in BAT **(Polak et al. 2008)**. However, the response of AdRaKO mice to cold exposure has not yet been tested. Thus, to investigate this, AdRaKO mice could be exposed to 4°C and their body temperature, energy expenditure and gene expression profile in BAT could be determined. Based on the current knowledge it could be that AdRaKO mice display an improved cold tolerance due to an enhanced thermogenic capacity in WAT. However, since we found that mTORC1 signaling is induced in BAT upon β-adrenergic stimulation, it could be that UCP1 induction in BAT upon cold exposure is impaired in AdRaKO mice, resulting in an impaired thermogenic response.

Taken together, the two studies presented in this thesis describe novel functions of hypothalamic and adipose mTOR signaling in the regulation of systemic energy homeostasis. Surprisingly, we found that mTORC1 signaling in Agrp neurons is dispensable for the regulation of food intake and organismal energetics. However, we demonstrate a novel role for mTORC2 signaling in cold-induced glucose uptake and glycolysis in BAT, which in turn is crucial for the regulation of systemic energy homeostasis upon cold stress.

Our results could thus be important for the development of novel therapies to treat metabolic disorders, such as diabetes and obesity. In particular, our findings imply that the use of mTOR inhibitors as an anti-obesity and anti-diabetic treatment could have potential caveats due to the impact of mTORC2 in BAT on temperature homeostasis and glucose metabolism. Moreover, our study suggests that glucose metabolism in BAT plays a major role for the maintenance of energy homeostasis under NST. Our data suggest that activation mTORC2 in BAT, to stimulate glucose metabolism, could have synergistic effects with NST activators in the treatment of obesity.

Chapter 5: References

5. References

Albert, V. and M. N. Hall (2014). "mTOR signaling in cellular and organismal energetics." Curr Opin Cell Biol **33C**: 55-66.

Anisimov, V. N., M. A. Zabezhinski, I. G. Popovich, T. S. Piskunova, A. V. Semenchenko, M. L. Tyndyk, M. N. Yurova, M. P. Antoch and M. V. Blagosklonny (2010). "Rapamycin extends maximal lifespan in cancer-prone mice." Am J Pathol **176**(5): 2092-2097.

Arora, S. and Anubhuti (2006). "Role of neuropeptides in appetite regulation and obesity--a review." Neuropeptides **40**(6): 375-401.

Backman, S. A., V. Stambolic, A. Suzuki, J. Haight, A. Elia, J. Pretorius, M. S. Tsao, P. Shannon, B. Bolon, G. O. Ivy and T. W. Mak (2001). "Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease." Nat Genet **29**(4): 396-403.

Ben-Sahra, I., J. J. Howell, J. M. Asara and B. D. Manning (2013). "Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1." Science **339**(6125): 1323-1328.

Benjamin, D., M. Colombi, C. Moroni and M. N. Hall (2011). "Rapamycin passes the torch: a new generation of mTOR inhibitors." Nat Rev Drug Discov **10**(11): 868-880.

Bentzinger, C. F., S. Lin, K. Romanino, P. Castets, M. Guridi, S. Summermatter, C. Handschin, L. A. Tintignac, M. N. Hall and M. A. Ruegg (2013). "Differential response of skeletal muscles to mTORC1 signaling during atrophy and hypertrophy." Skelet Muscle **3**(1): 6.

Bentzinger, C. F., K. Romanino, D. Cloetta, S. Lin, J. B. Mascarenhas, F. Oliveri, J. Xia, E. Casanova, C. F. Costa, M. Brink, F. Zorzato, M. N. Hall and M. A. Ruegg (2008). "Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy." Cell Metab **8**(5): 411-424.

Betz, C., D. Stracka, C. Prescianotto-Baschong, M. Frieden, N. Demaurex and M. N. Hall (2013). "Feature Article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology." Proc Natl Acad Sci U S A **110**(31): 12526-12534.

Bjedov, I., J. M. Toivonen, F. Kerr, C. Slack, J. Jacobson, A. Foley and L. Partridge (2010). "Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster." Cell Metab **11**(1): 35-46.

Bookout, A. L., M. H. de Groot, B. M. Owen, S. Lee, L. Gautron, H. L. Lawrence, X. Ding, J. K. Elmquist, J. S. Takahashi, D. J. Mangelsdorf and S. A. Kliewer (2013).

"FGF21 regulates metabolism and circadian behavior by acting on the nervous system." Nat Med **19**(9): 1147-1152.

Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane and S. L. Schreiber (1994). "A mammalian protein targeted by G1-arresting rapamycin-receptor complex." Nature **369**(6483): 756-758.

Busiello, R. A., S. Savarese and A. Lombardi (2015). "Mitochondrial uncoupling proteins and energy metabolism." Front Physiol **6**: 36.

Cannon, B. and J. Nedergaard (2004). "Brown adipose tissue: function and physiological significance." Physiol Rev **84**(1): 277-359.

Cao, R., F. E. Anderson, Y. J. Jung, H. Dziema and K. Obrietan (2011). "Circadian regulation of mammalian target of rapamycin signaling in the mouse suprachiasmatic nucleus." Neuroscience **181**: 79-88.

Castets, P., S. Lin, N. Rion, S. Di Fulvio, K. Romanino, M. Guridi, S. Frank, L. A. Tintignac, M. Sinnreich and M. A. Ruegg (2013). "Sustained activation of mTORC1 in skeletal muscle inhibits constitutive and starvation-induced autophagy and causes a severe, late-onset myopathy." Cell Metab **17**(5): 731-744.

Chauvin, C., V. Koka, A. Nouschi, V. Mieulet, C. Hoareau-Aveilla, A. Dreazen, N. Cagnard, W. Carpentier, T. Kiss, O. Meyuhas and M. Pende (2014). "Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program." Oncogene **33**(4): 474-483.

Chen, D., E. L. Thomas and P. Kapahi (2009). "HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in Caenorhabditis elegans." PLoS Genet **5**(5): e1000486.

Clapham, J. C. and J. R. Arch (2011). "Targeting thermogenesis and related pathways in anti-obesity drug discovery." Pharmacol Ther **131**(3): 295-308.

Cone, R. D., M. A. Cowley, A. A. Butler, W. Fan, D. L. Marks and M. J. Low (2001). "The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis." Int J Obes Relat Metab Disord **25 Suppl 5**: S63-67.

Considine, R. V., M. K. Sinha, M. L. Heiman, A. Kriauciunas, T. W. Stephens, M. R. Nyce, J. P. Ohannesian, C. C. Marco, L. J. McKee, T. L. Bauer and et al. (1996). "Serum immunoreactive-leptin concentrations in normal-weight and obese humans." N Engl J Med **334**(5): 292-295.

Cornu, M., W. Oppliger, V. Albert, A. M. Robitaille, F. Trapani, L. Quagliata, T. Fuhrer, U. Sauer, L. Terracciano and M. N. Hall (2014). "Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21." Proc Natl Acad Sci U S A **111**(32): 11592-11599.

Corradetti, M. N., K. Inoki, N. Bardeesy, R. A. DePinho and K. L. Guan (2004). "Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome." Genes Dev **18**(13): 1533-1538.

Cota, D., K. Proulx, K. A. Smith, S. C. Kozma, G. Thomas, S. C. Woods and R. J. Seeley (2006). "Hypothalamic mTOR signaling regulates food intake." Science **312**(5775): 927-930.

Curatolo, P., R. Bombardieri and S. Jozwiak (2008). "Tuberous sclerosis." Lancet **372**(9639): 657-668.

Cybulski, N. and M. N. Hall (2009). "TOR complex 2: a signaling pathway of its own." Trends Biochem Sci **34**(12): 620-627.

Cybulski, N., P. Polak, J. Auwerx, M. A. Ruegg and M. N. Hall (2009). "mTOR complex 2 in adipose tissue negatively controls whole-body growth." Proc Natl Acad Sci U S A **106**(24): 9902-9907.

Cypess, A. M., S. Lehman, G. Williams, I. Tal, D. Rodman, A. B. Goldfine, F. C. Kuo, E. L. Palmer, Y. H. Tseng, A. Doria, G. M. Kolodny and C. R. Kahn (2009). "Identification and importance of brown adipose tissue in adult humans." N Engl J Med **360**(15): 1509-1517.

Dallner, O. S., E. Chernogubova, K. A. Brolinson and T. Bengtsson (2006). "Beta3-adrenergic receptors stimulate glucose uptake in brown adipocytes by two mechanisms independently of glucose transporter 4 translocation." Endocrinology **147**(12): 5730-5739.

Dan, H. C., M. J. Cooper, P. C. Cogswell, J. A. Duncan, J. P. Ting and A. S. Baldwin (2008). "Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK." Genes Dev **22**(11): 1490-1500.

Dan, H. C., M. Sun, L. Yang, R. I. Feldman, X. M. Sui, C. C. Ou, M. Nellist, R. S. Yeung, D. J. Halley, S. V. Nicosia, W. J. Pledger and J. Q. Cheng (2002). "Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin." J Biol Chem **277**(38): 35364-35370.

Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh and M. E. Greenberg (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery." Cell **91**(2): 231-241.

Dazert, E. and M. N. Hall (2011). "mTOR signaling in disease." Curr Opin Cell Biol **23**(6): 744-755.

de Joussineau, C., I. Sahut-Barnola, F. Tissier, T. Dumontet, C. Drelon, M. Batisse-Lignier, I. Tauveron, J. C. Pointud, A. M. Lefrancois-Martinez, C. A. Stratakis, J. Bertherat, P. Val and A. Martinez (2014). "mTOR pathway is activated by PKA in

adrenocortical cells and participates in vivo to apoptosis resistance in primary pigmented nodular adrenocortical disease (PPNAD)." Hum Mol Genet **23**(20): 5418-5428.

De Meis, L., L. A. Ketzer, J. Camacho-Pereira and A. Galina (2012). "Brown adipose tissue mitochondria: modulation by GDP and fatty acids depends on the respiratory substrates." Biosci Rep **32**(1): 53-59.

Dejardin, J. and R. E. Kingston (2009). "Purification of proteins associated with specific genomic Loci." Cell **136**(1): 175-186.

Di Cristofano, A., B. Pesce, C. Cordon-Cardo and P. P. Pandolfi (1998). "Pten is essential for embryonic development and tumour suppression." Nat Genet **19**(4): 348-355.

Dibble, C. C. and B. D. Manning (2013). "Signal integration by mTORC1 coordinates nutrient input with biosynthetic output." Nat Cell Biol **15**(6): 555-564.

Dijkers, P. F., K. U. Birkenkamp, E. W. Lam, N. S. Thomas, J. W. Lammers, L. Koenderman and P. J. Coffer (2002). "FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity." J Cell Biol **156**(3): 531-542.

Dijkers, P. F., R. H. Medema, J. W. Lammers, L. Koenderman and P. J. Coffer (2000). "Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1." Curr Biol **10**(19): 1201-1204.

Duncan, R. E., M. Ahmadian, K. Jaworski, E. Sarkadi-Nagy and H. S. Sul (2007). "Regulation of lipolysis in adipocytes." Annu Rev Nutr **27**: 79-101.

Duvel, K., J. L. Yecies, S. Menon, P. Raman, A. I. Lipovsky, A. L. Souza, E. Triantafellow, Q. Ma, R. Gorski, S. Cleaver, M. G. Vander Heiden, J. P. MacKeigan, P. M. Finan, C. B. Clish, L. O. Murphy and B. D. Manning (2010). "Activation of a metabolic gene regulatory network downstream of mTOR complex 1." Mol Cell **39**(2): 171-183.

Eberle, D., B. Hegarty, P. Bossard, P. Ferre and F. Foufelle (2004). "SREBP transcription factors: master regulators of lipid homeostasis." Biochimie **86**(11): 839-848.

Egan, B. and J. R. Zierath (2013). "Exercise metabolism and the molecular regulation of skeletal muscle adaptation." Cell Metab **17**(2): 162-184.

Erickson, J. C., K. E. Clegg and R. D. Palmiter (1996). "Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y." Nature **381**(6581): 415-421.

Ernst, M. B., C. M. Wunderlich, S. Hess, M. Paehler, A. Mesaros, S. B. Koralov, A. Kleinridders, A. Husch, H. Munzberg, B. Hampel, J. Alber, P. Kloppenburg, J. C. Bruning and F. T. Wunderlich (2009). "Enhanced Stat3 activation in POMC neurons provokes negative feedback inhibition of leptin and insulin signaling in obesity." J Neurosci **29**(37): 11582-11593.

Facchinetti, V., W. Ouyang, H. Wei, N. Soto, A. Lazorchak, C. Gould, C. Lowry, A. C. Newton, Y. Mao, R. Q. Miao, W. C. Sessa, J. Qin, P. Zhang, B. Su and E. Jacinto (2008). "The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C." EMBO J **27**(14): 1932-1943.

Fetissov, S. O., J. Kopp and T. Hokfelt (2004). "Distribution of NPY receptors in the hypothalamus." Neuropeptides **38**(4): 175-188.

Freeman, D., R. Lesche, N. Kertesz, S. Wang, G. Li, J. Gao, M. Groszer, H. Martinez-Diaz, N. Rozengurt, G. Thomas, X. Liu and H. Wu (2006). "Genetic background controls tumor development in PTEN-deficient mice." Cancer Res **66**(13): 6492-6496.

Frias, M. A., C. C. Thoreen, J. D. Jaffe, W. Schroder, T. Sculley, S. A. Carr and D. M. Sabatini (2006). "mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s." Curr Biol **16**(18): 1865-1870.

Frontini, A. and S. Cinti (2010). "Distribution and development of brown adipocytes in the murine and human adipose organ." Cell Metab **11**(4): 253-256.

Ganley, I. G., H. Lam du, J. Wang, X. Ding, S. Chen and X. Jiang (2009). "ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy." J Biol Chem **284**(18): 12297-12305.

Garcia-Martinez, J. M. and D. R. Alessi (2008). "mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1)." Biochem J **416**(3): 375-385.

Gaubitz, C., T. M. Oliveira, M. Prouteau, A. Leitner, M. Karuppasamy, G. Konstantinidou, D. Rispal, S. Eltschinger, G. C. Robinson, S. Thore, R. Aebersold, C. Schaffitzel and R. Loewith (2015). "Molecular Basis of the Rapamycin Insensitivity of Target Of Rapamycin Complex 2." Mol Cell.

Gehlert, D. R. (2004). "Introduction to the reviews on neuropeptide Y." Neuropeptides **38**(4): 135-140.

Gilley, J., P. J. Coffer and J. Ham (2003). "FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons." J Cell Biol **162**(4): 613-622.

Gottlob, K., N. Majewski, S. Kennedy, E. Kandel, R. B. Robey and N. Hay (2001). "Inhibition of early apoptotic events by Akt/PKB is dependent on the first

committed step of glycolysis and mitochondrial hexokinase." Genes Dev **15**(11): 1406-1418.

Greco-Perotto, R., D. Zaninetti, F. Assimacopoulos-Jeannet, E. Bobbioni and B. Jeanrenaud (1987). "Stimulatory effect of cold adaptation on glucose utilization by brown adipose tissue. Relationship with changes in the glucose transporter system." J Biol Chem **262**(16): 7732-7736.

Greenman, Y., N. Golani, S. Gilad, M. Yaron, R. Limor and N. Stern (2004). "Ghrelin secretion is modulated in a nutrient- and gender-specific manner." Clin Endocrinol (Oxf) **60**(3): 382-388.

Guertin, D. A., D. M. Stevens, M. Saitoh, S. Kinkel, K. Crosby, J. H. Sheen, D. J. Mullholland, M. A. Magnuson, H. Wu and D. M. Sabatini (2009). "mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice." Cancer Cell **15**(2): 148-159.

Gwinn, D. M., D. B. Shackelford, D. F. Egan, M. M. Mihaylova, A. Mery, D. S. Vasquez, B. E. Turk and R. J. Shaw (2008). "AMPK phosphorylation of raptor mediates a metabolic checkpoint." Mol Cell **30**(2): 214-226.

Haemmerle, G., A. Lass, R. Zimmermann, G. Gorkiewicz, C. Meyer, J. Rozman, G. Heldmaier, R. Maier, C. Theussl, S. Eder, D. Kratky, E. F. Wagner, M. Klingenspor, G. Hoefler and R. Zechner (2006). "Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase." Science **312**(5774): 734-737.

Hagiwara, A., M. Cornu, N. Cybulski, P. Polak, C. Betz, F. Trapani, L. Terracciano, M. H. Heim, M. A. Ruegg and M. N. Hall (2012). "Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c." Cell Metab **15**(5): 725-738.

Hao, Q., R. Yadav, A. L. Basse, S. Petersen, S. B. Sonne, S. Rasmussen, Q. Zhu, Z. Lu, J. Wang, K. Audouze, R. Gupta, L. Madsen, K. Kristiansen and J. B. Hansen (2015). "Transcriptome profiling of brown adipose tissue during cold exposure reveals extensive regulation of glucose metabolism." Am J Physiol Endocrinol Metab **308**(5): E380-392.

Hardie, D. G. and S. A. Hawley (2001). "AMP-activated protein kinase: the energy charge hypothesis revisited." Bioessays **23**(12): 1112-1119.

Harms, M. and P. Seale (2013). "Brown and beige fat: development, function and therapeutic potential." Nat Med **19**(10): 1252-1263.

Harrington, L. S., G. M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N. R. Leslie, S. Cheng, P. R. Shepherd, I. Gout, C. P. Downes and R. F. Lamb (2004). "The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins." J Cell Biol **166**(2): 213-223.

Harrington, L. S., G. M. Findlay and R. F. Lamb (2005). "Restraining PI3K: mTOR signalling goes back to the membrane." Trends Biochem Sci **30**(1): 35-42.

Harrison, D. E., R. Strong, Z. D. Sharp, J. F. Nelson, C. M. Astle, K. Flurkey, N. L. Nadon, J. E. Wilkinson, K. Frenkel, C. S. Carter, M. Pahor, M. A. Javors, E. Fernandez and R. A. Miller (2009). "Rapamycin fed late in life extends lifespan in genetically heterogeneous mice." Nature **460**(7253): 392-395.

Haugen, F. and C. A. Drevon (2007). "The interplay between nutrients and the adipose tissue." Proc Nutr Soc **66**(2): 171-182.

Havrankova, J., J. Roth and M. Brownstein (1978). "Insulin receptors are widely distributed in the central nervous system of the rat." Nature **272**(5656): 827-829.

Heitman, J., N. R. Movva and M. N. Hall (1991). "Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast." Science **253**(5022): 905-909.

Horie, Y., A. Suzuki, E. Kataoka, T. Sasaki, K. Hamada, J. Sasaki, K. Mizuno, G. Hasegawa, H. Kishimoto, M. Iizuka, M. Naito, K. Enomoto, S. Watanabe, T. W. Mak and T. Nakano (2004). "Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas." J Clin Invest **113**(12): 1774-1783.

Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S. Iemura, T. Natsume, K. Takehana, N. Yamada, J. L. Guan, N. Oshiro and N. Mizushima (2009). "Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy." Mol Biol Cell **20**(7): 1981-1991.

Hotamisligil, G. S., N. S. Shargill and B. M. Spiegelman (1993). "Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance." Science **259**(5091): 87-91.

Hresko, R. C. and M. Mueckler (2005). "mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes." J Biol Chem **280**(49): 40406-40416.

Hung, C. M., C. M. Calejman, J. Sanchez-Gurmaches, H. Li, C. B. Clish, S. Hettmer, A. J. Wagers and D. A. Guertin (2014). "Rictor/mTORC2 loss in the Myf5 lineage reprograms brown fat metabolism and protects mice against obesity and metabolic disease." Cell Rep **8**(1): 256-271.

Ikenoue, T., K. Inoki, Q. Yang, X. Zhou and K. L. Guan (2008). "Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling." EMBO J **27**(14): 1919-1931.

Inoki, K., Y. Li, T. Zhu, J. Wu and K. L. Guan (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." Nat Cell Biol **4**(9): 648-657.

Inoki, K., H. Ouyang, T. Zhu, C. Lindvall, Y. Wang, X. Zhang, Q. Yang, C. Bennett, Y. Harada, K. Stankunas, C. Y. Wang, X. He, O. A. MacDougald, M. You, B. O. Williams and K. L. Guan (2006). "TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth." Cell **126**(5): 955-968.

Jacinto, E., V. Facchinetti, D. Liu, N. Soto, S. Wei, S. Y. Jung, Q. Huang, J. Qin and B. Su (2006). "SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity." Cell **127**(1): 125-137.

Jacinto, E., R. Loewith, A. Schmidt, S. Lin, M. A. Ruegg, A. Hall and M. N. Hall (2004). "Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive." Nat Cell Biol **6**(11): 1122-1128.

Jacinto, E. and A. Lorberg (2008). "TOR regulation of AGC kinases in yeast and mammals." Biochem J **410**(1): 19-37.

Jimenez, V., S. Munoz, E. Casana, C. Mallol, I. Elias, C. Jambrina, A. Ribera, T. Ferre, S. Franckhauser and F. Bosch (2013). "In vivo adeno-associated viral vector-mediated genetic engineering of white and brown adipose tissue in adult mice." Diabetes **62**(12): 4012-4022.

Jouffe, C., G. Cretenet, L. Symul, E. Martin, F. Atger, F. Naef and F. Gachon (2013). "The circadian clock coordinates ribosome biogenesis." PLoS Biol **11**(1): e1001455.

Jung, C. H., C. B. Jun, S. H. Ro, Y. M. Kim, N. M. Otto, J. Cao, M. Kundu and D. H. Kim (2009). "ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery." Mol Biol Cell **20**(7): 1992-2003.

Kaeberlein, M., R. W. Powers, 3rd, K. K. Steffen, E. A. Westman, D. Hu, N. Dang, E. O. Kerr, K. T. Kirkland, S. Fields and B. K. Kennedy (2005). "Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients." Science **310**(5751): 1193-1196.

Kajimura, S., P. Seale, T. Tomaru, H. Erdjument-Bromage, M. P. Cooper, J. L. Ruas, S. Chin, P. Tempst, M. A. Lazar and B. M. Spiegelman (2008). "Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex." Genes Dev **22**(10): 1397-1409.

Kamei, N., K. Tobe, R. Suzuki, M. Ohsugi, T. Watanabe, N. Kubota, N. Ohtsuka-Kowatari, K. Kumagai, K. Sakamoto, M. Kobayashi, T. Yamauchi, K. Ueki, Y. Oishi, S. Nishimura, I. Manabe, H. Hashimoto, Y. Ohnishi, H. Ogata, K. Tokuyama, M. Tsunoda, T. Ide, K. Murakami, R. Nagai and T. Kadowaki (2006). "Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance." J Biol Chem **281**(36): 26602-26614.

Kanda, H., S. Tateya, Y. Tamori, K. Kotani, K. Hiasa, R. Kitazawa, S. Kitazawa, H. Miyachi, S. Maeda, K. Egashira and M. Kasuga (2006). "MCP-1 contributes to

macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity." J Clin Invest **116**(6): 1494-1505.

Kane, S., H. Sano, S. C. Liu, J. M. Asara, W. S. Lane, C. C. Garner and G. E. Lienhard (2002). "A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain." J Biol Chem **277**(25): 22115-22118.

Kanneganti, T. D. and V. D. Dixit (2012). "Immunological complications of obesity." Nat Immunol **13**(8): 707-712.

Kapahi, P., B. M. Zid, T. Harper, D. Koslover, V. Sapin and S. Benzer (2004). "Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway." Curr Biol **14**(10): 885-890.

Keith, C. T. and S. L. Schreiber (1995). "PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints." Science **270**(5233): 50-51.

Kenerson, H. L., M. M. Yeh, M. Kazami, X. Jiang, K. J. Riehle, R. L. McIntyre, J. O. Park, S. Kwon, J. S. Campbell and R. S. Yeung (2013). "Akt and mTORC1 have different roles during liver tumorigenesis in mice." Gastroenterology **144**(5): 1055-1065.

Kenerson, H. L., M. M. Yeh and R. S. Yeung (2011). "Tuberous sclerosis complex-1 deficiency attenuates diet-induced hepatic lipid accumulation." PLoS One **6**(3): e18075.

Khapre, R. V., S. A. Patel, A. A. Kondratova, A. Chaudhary, N. Velingkaar, M. P. Antoch and R. V. Kondratov (2014). "Metabolic clock generates nutrient anticipation rhythms in mTOR signaling." Aging (Albany NY) **6**(8): 675-689.

Kim, E., P. Goraksha-Hicks, L. Li, T. P. Neufeld and K. L. Guan (2008). "Regulation of TORC1 by Rag GTPases in nutrient response." Nat Cell Biol **10**(8): 935-945.

Kim, J., M. Kundu, B. Viollet and K. L. Guan (2011). "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1." Nat Cell Biol **13**(2): 132-141.

Kitamura, T., Y. Feng, Y. I. Kitamura, S. C. Chua, Jr., A. W. Xu, G. S. Barsh, L. Rossetti and D. Accili (2006). "Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake." Nat Med **12**(5): 534-540.

Klaus, S., L. Casteilla, F. Bouillaud and D. Ricquier (1991). "The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue." Int J Biochem **23**(9): 791-801.

Klein, J., M. Fasshauer, H. H. Klein, M. Benito and C. R. Kahn (2002). "Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action." Bioessays **24**(4): 382-388.

Kocalis, H. E., S. L. Hagan, L. George, M. K. Turney, M. A. Siuta, G. N. Laryea, L. C. Morris, L. J. Muglia, R. L. Printz, G. D. Stanwood and K. D. Niswender (2014). "Rictor/mTORC2 facilitates central regulation of energy and glucose homeostasis." Mol Metab **3**(4): 394-407.

Komatsu, M., M. Takei, H. Ishii and Y. Sato (2013). "Glucose-stimulated insulin secretion: A newer perspective." J Diabetes Investig **4**(6): 511-516.

Krude, H., D. Schnabel, W. Luck and A. Gruters (1999). "Implications of the phenotype of POMC deficiency for the role of POMC-derived peptides in skin physiology." Ann N Y Acad Sci **885**: 419-421.

Kumar, A., T. E. Harris, S. R. Keller, K. M. Choi, M. A. Magnuson and J. C. Lawrence, Jr. (2008). "Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity." Mol Cell Biol **28**(1): 61-70.

Kumar, A., J. C. Lawrence, Jr., D. Y. Jung, H. J. Ko, S. R. Keller, J. K. Kim, M. A. Magnuson and T. E. Harris (2010). "Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism." Diabetes **59**(6): 1397-1406.

Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva and M. N. Hall (1993). "Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression." Cell **73**(3): 585-596.

Kwiatkowski, D. J., H. Zhang, J. L. Bandura, K. M. Heiberger, M. Glogauer, N. el-Hashemite and H. Onda (2002). "A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells." Hum Mol Genet **11**(5): 525-534.

Lago, F., C. Dieguez, J. Gomez-Reino and O. Gualillo (2007). "The emerging role of adipokines as mediators of inflammation and immune responses." Cytokine Growth Factor Rev **18**(3-4): 313-325.

Laplante, M. and D. M. Sabatini (2012). "mTOR signaling in growth control and disease." Cell **149**(2): 274-293.

Laviola, L., A. Natalicchio and F. Giorgino (2007). "The IGF-I signaling pathway." Curr Pharm Des **13**(7): 663-669.

Levin, B. E., C. Magnan, A. Dunn-Meynell and C. Le Foll (2011). "Metabolic sensing and the brain: who, what, where, and how?" Endocrinology **152**(7): 2552-2557.

Li, S., M. S. Brown and J. L. Goldstein (2010). "Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis." Proc Natl Acad Sci U S A **107**(8): 3441-3446.

Lindberg, O., J. de Pierre, E. Rylander and B. A. Afzelius (1967). "Studies of the mitochondrial energy-transfer system of brown adipose tissue." J Cell Biol **34**(1): 293-310.

Liu, M., J. Bai, S. He, R. Villarreal, D. Hu, C. Zhang, X. Yang, H. Liang, T. J. Slaga, Y. Yu, Z. Zhou, J. Blenis, P. E. Scherer, L. Q. Dong and F. Liu (2014). "Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1." Cell Metab **19**(6): 967-980.

Loewith, R. and M. N. Hall (2011). "Target of rapamycin (TOR) in nutrient signaling and growth control." Genetics **189**(4): 1177-1201.

Loncar, D. (1991). "Convertible adipose tissue in mice." Cell Tissue Res **266**(1): 149-161.

Loncar, D., B. A. Afzelius and B. Cannon (1988). "Epididymal white adipose tissue after cold stress in rats. I. Nonmitochondrial changes." J Ultrastruct Mol Struct Res **101**(2-3): 109-122.

Long, X., Y. Lin, S. Ortiz-Vega, K. Yonezawa and J. Avruch (2005). "Rheb binds and regulates the mTOR kinase." Curr Biol **15**(8): 702-713.

Lumeng, C. N., J. L. Bodzin and A. R. Saltiel (2007). "Obesity induces a phenotypic switch in adipose tissue macrophage polarization." J Clin Invest **117**(1): 175-184.

Lumeng, C. N., S. M. Deyoung, J. L. Bodzin and A. R. Saltiel (2007). "Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity." Diabetes **56**(1): 16-23.

Luquet, S. and C. Magnan (2009). "The central nervous system at the core of the regulation of energy homeostasis." Front Biosci (Schol Ed) **1**: 448-465.

Luquet, S., F. A. Perez, T. S. Hnasko and R. D. Palmiter (2005). "NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates." Science **310**(5748): 683-685.

Lynch, G. S. and J. G. Ryall (2008). "Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease." Physiol Rev **88**(2): 729-767.

Ma, L., J. Teruya-Feldstein, P. Bonner, R. Bernardi, D. N. Franz, D. Witte, C. Cordon-Cardo and P. P. Pandolfi (2007). "Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer." Cancer Res **67**(15): 7106-7112.

Ma, X. M. and J. Blenis (2009). "Molecular mechanisms of mTOR-mediated translational control." Nat Rev Mol Cell Biol **10**(5): 307-318.

Madamanchi, A. (2007). "Beta-adrenergic receptor signaling in cardiac function and heart failure." Mcgill J Med **10**(2): 99-104.

Manning, B. D. and L. C. Cantley (2007). "AKT/PKB signaling: navigating downstream." Cell **129**(7): 1261-1274.

Manning, B. D., A. R. Tee, M. N. Logsdon, J. Blenis and L. C. Cantley (2002). "Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway." Mol Cell **10**(1): 151-162.

Mao, X., Y. Fujiwara, A. Chapdelaine, H. Yang and S. H. Orkin (2001). "Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain." Blood **97**(1): 324-326.

Marsh, D. J., G. Hollopeter, K. E. Kafer and R. D. Palmiter (1998). "Role of the Y5 neuropeptide Y receptor in feeding and obesity." Nat Med **4**(6): 718-721.

Matsuzaki, H., H. Daitoku, M. Hatta, K. Tanaka and A. Fukamizu (2003). "Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation." Proc Natl Acad Sci U S A **100**(20): 11285-11290.

Mei, F. C., J. Qiao, O. M. Tsygankova, J. L. Meinkoth, L. A. Quilliam and X. Cheng (2002). "Differential signaling of cyclic AMP: opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation." J Biol Chem **277**(13): 11497-11504.

Meikle, L., J. R. McMullen, M. C. Sherwood, A. S. Lader, V. Walker, J. A. Chan and D. J. Kwiatkowski (2005). "A mouse model of cardiac rhabdomyoma generated by loss of Tsc1 in ventricular myocytes." Hum Mol Genet **14**(3): 429-435.

Meikle, L., D. M. Talos, H. Onda, K. Pollizzi, A. Rotenberg, M. Sahin, F. E. Jensen and D. J. Kwiatkowski (2007). "A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival." J Neurosci **27**(21): 5546-5558.

Meister, B. (2000). "Control of food intake via leptin receptors in the hypothalamus." Vitam Horm **59**: 265-304.

Menendez, J. A. and R. Lupu (2007). "Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis." Nat Rev Cancer **7**(10): 763-777.

Menon, S., J. L. Yecies, H. H. Zhang, J. J. Howell, J. Nicholatos, E. Harputlugil, R. T. Bronson, D. J. Kwiatkowski and B. D. Manning (2012). "Chronic activation of mTOR complex 1 is sufficient to cause hepatocellular carcinoma in mice." Sci Signal **5**(217): ra24.

Mesaros, A., S. B. Koralov, E. Rother, F. T. Wunderlich, M. B. Ernst, G. S. Barsh, K. Rajewsky and J. C. Bruning (2008). "Activation of Stat3 signaling in AgRP neurons promotes locomotor activity." Cell Metab **7**(3): 236-248.

Miller, R. A., D. E. Harrison, C. M. Astle, J. A. Baur, A. R. Boyd, R. de Cabo, E. Fernandez, K. Flurkey, M. A. Javors, J. F. Nelson, C. J. Orihuela, S. Pletcher, Z. D. Sharp, D. Sinclair, J. W. Starnes, J. E. Wilkinson, N. L. Nadon and R. Strong (2011). "Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice." J Gerontol A Biol Sci Med Sci **66**(2): 191-201.

Mills, C. D. (2012). "M1 and M2 Macrophages: Oracles of Health and Disease." Crit Rev Immunol **32**(6): 463-488.

Misra, U. K. and S. V. Pizzo (2012). "Upregulation of mTORC2 activation by the selective agonist of EPAC, 8-CPT-2Me-cAMP, in prostate cancer cells: assembly of a multiprotein signaling complex." J Cell Biochem **113**(5): 1488-1500.

Miyamoto, S., A. N. Murphy and J. H. Brown (2008). "Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II." Cell Death Differ **15**(3): 521-529.

Mori, H., K. Inoki, H. Munzberg, D. Opland, M. Faouzi, E. C. Villanueva, T. Ikenoue, D. Kwiatkowski, O. A. MacDougald, M. G. Myers, Jr. and K. L. Guan (2009). "Critical role for hypothalamic mTOR activity in energy balance." Cell Metab **9**(4): 362-374.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa and S. Matsukura (2001). "A role for ghrelin in the central regulation of feeding." Nature **409**(6817): 194-198.

Neary, N. M., A. P. Goldstone and S. R. Bloom (2004). "Appetite regulation: from the gut to the hypothalamus." Clin Endocrinol (Oxf) **60**(2): 153-160.

Ng, M., T. Fleming, M. Robinson, B. Thomson, N. Graetz, C. Margono, E. C. Mullany, S. Biryukov, C. Abbafati, S. F. Abera, J. P. Abraham, N. M. Abu-Rmeileh, T. Achoki, F. S. AlBuhairan, Z. A. Alemu, R. Alfonso, M. K. Ali, R. Ali, N. A. Guzman, W. Ammar, P. Anwari, A. Banerjee, S. Barquera, S. Basu, D. A. Bennett, Z. Bhutta, J. Blore, N. Cabral, I. C. Nonato, J. C. Chang, R. Chowdhury, K. J. Courville, M. H. Criqui, D. K. Cundiff, K. C. Dabhadkar, L. Dandona, A. Davis, A. Dayama, S. D. Dharmaratne, E. L. Ding, A. M. Durrani, A. Esteghamati, F. Farzadfar, D. F. Fay, V. L. Feigin, A. Flaxman, M. H. Forouzanfar, A. Goto, M. A. Green, R. Gupta, N. Hafezi-Nejad, G. J. Hankey, H. C. Harewood, R. Havmoeller, S. Hay, L. Hernandez, A. Husseini, B. T. Idrisov, N. Ikeda, F. Islami, E. Jahangir, S. K. Jassal, S. H. Jee, M. Jeffreys, J. B. Jonas, E. K. Kabagambe, S. E. Khalifa, A. P. Kengne, Y. S. Khader, Y. H. Khang, D. Kim, R. W. Kimokoti, J. M. Kinge, Y. Kokubo, S. Kosen, G. Kwan, T. Lai, M. Leinsalu, Y. Li, X. Liang, S. Liu, G. Logroscino, P. A. Lotufo, Y. Lu, J. Ma, N. K. Mainoo, G. A. Mensah, T. R. Merriman, A. H. Mokdad, J. Moschandreas, M. Naghavi, A. Naheed, D. Nand, K. M. Narayan, E. L. Nelson, M. L. Neuhouser, M. I. Nisar, T. Ohkubo, S. O. Oti, A.

Pedroza, D. Prabhakaran, N. Roy, U. Sampson, H. Seo, S. G. Sepanlou, K. Shibuya, R. Shiri, I. Shiue, G. M. Singh, J. A. Singh, V. Skirbekk, N. J. Stapelberg, L. Sturua, B. L. Sykes, M. Tobias, B. X. Tran, L. Trasande, H. Toyoshima, S. van de Vijver, T. J. Vasankari, J. L. Veerman, G. Velasquez-Melendez, V. V. Vlassov, S. E. Vollset, T. Vos, C. Wang, X. Wang, E. Weiderpass, A. Werdecker, J. L. Wright, Y. C. Yang, H. Yatsuya, J. Yoon, S. J. Yoon, Y. Zhao, M. Zhou, S. Zhu, A. D. Lopez, C. J. Murray and E. Gakidou (2014). "Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013." Lancet **384**(9945): 766-781.

Nicholls, D. G. (1974). "Hamster brown-adipose-tissue mitochondria. The control of respiration and the proton electrochemical potential gradient by possible physiological effectors of the proton conductance of the inner membrane." Eur J Biochem **49**(3): 573-583.

Olsen, J. M., M. Sato, O. S. Dallner, A. L. Sandstrom, D. F. Pisani, J. C. Chambard, E. Z. Amri, D. S. Hutchinson and T. Bengtsson (2014). "Glucose uptake in brown fat cells is dependent on mTOR complex 2-promoted GLUT1 translocation." J Cell Biol **207**(3): 365-374.

Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer and D. B. Donner (1999). "NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase." Nature **401**(6748): 82-85.

Palkovits, M. (1973). "Isolated removal of hypothalamic or other brain nuclei of the rat." Brain Res **59**: 449-450.

Palmada, M., C. Boehmer, A. Akel, J. Rajamanickam, S. Jeyaraj, K. Keller and F. Lang (2006). "SGK1 kinase upregulates GLUT1 activity and plasma membrane expression." Diabetes **55**(2): 421-427.

Palmiter, R. D., J. C. Erickson, G. Hollopeter, S. C. Baraban and M. W. Schwartz (1998). "Life without neuropeptide Y." Recent Prog Horm Res **53**: 163-199.

Penicaud, L., B. Cousin, C. Leloup, A. Lorsignol and L. Casteilla (2000). "The autonomic nervous system, adipose tissue plasticity, and energy balance." Nutrition **16**(10): 903-908.

Perry, J. and N. Kleckner (2003). "The ATRs, ATMs, and TORs are giant HEAT repeat proteins." Cell **112**(2): 151-155.

Peschiaroli, A., N. V. Dorrello, D. Guardavaccaro, M. Venere, T. Halazonetis, N. E. Sherman and M. Pagano (2006). "SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response." Mol Cell **23**(3): 319-329.

Peterson, T. R., S. S. Sengupta, T. E. Harris, A. E. Carmack, S. A. Kang, E. Balderas, D. A. Guertin, K. L. Madden, A. E. Carpenter, B. N. Finck and D. M. Sabatini (2011).

"mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway." Cell **146**(3): 408-420.

Plum, L., B. F. Belgardt and J. C. Bruning (2006). "Central insulin action in energy and glucose homeostasis." J Clin Invest **116**(7): 1761-1766.

Plum, L., H. V. Lin, R. Dutia, J. Tanaka, K. S. Aizawa, M. Matsumoto, A. J. Kim, N. X. Cawley, J. H. Paik, Y. P. Loh, R. A. DePinho, S. L. Wardlaw and D. Accili (2009). "The obesity susceptibility gene Cpe links FoxO1 signaling in hypothalamic pro-opiomelanocortin neurons with regulation of food intake." Nat Med **15**(10): 1195-1201.

Podsypanina, K., L. H. Ellenson, A. Nemes, J. Gu, M. Tamura, K. M. Yamada, C. Cordon-Cardo, G. Catoretti, P. E. Fisher and R. Parsons (1999). "Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems." Proc Natl Acad Sci U S A **96**(4): 1563-1568.

Polak, P., N. Cybulski, J. N. Feige, J. Auwerx, M. A. Ruegg and M. N. Hall (2008). "Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration." Cell Metab **8**(5): 399-410.

Porstmann, T., C. R. Santos, B. Griffiths, M. Cully, M. Wu, S. Leevers, J. R. Griffiths, Y. L. Chung and A. Schulze (2008). "SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth." Cell Metab **8**(3): 224-236.

Potter, C. J., L. G. Pedraza and T. Xu (2002). "Akt regulates growth by directly phosphorylating Tsc2." Nat Cell Biol **4**(9): 658-665.

Powers, R. W., 3rd, M. Kaeberlein, S. D. Caldwell, B. K. Kennedy and S. Fields (2006). "Extension of chronological life span in yeast by decreased TOR pathway signaling." Genes Dev **20**(2): 174-184.

Pritchard, L. E., A. V. Turnbull and A. White (2002). "Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity." J Endocrinol **172**(3): 411-421.

Qian, S., H. Chen, D. Weingarth, M. E. Trumbauer, D. E. Novi, X. Guan, H. Yu, Z. Shen, Y. Feng, E. Frazier, A. Chen, R. E. Camacho, L. P. Shearman, S. Gopal-Truter, D. J. MacNeil, L. H. Van der Ploeg and D. J. Marsh (2002). "Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice." Mol Cell Biol **22**(14): 5027-5035.

Raught, B., F. Peiretti, A. C. Gingras, M. Livingstone, D. Shahbazian, G. L. Mayeur, R. D. Polakiewicz, N. Sonenberg and J. W. Hershey (2004). "Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases." EMBO J **23**(8): 1761-1769.

Rial, E., A. Poustie and D. G. Nicholls (1983). "Brown-adipose-tissue mitochondria: the regulation of the 32000-Mr uncoupling protein by fatty acids and purine nucleotides." Eur J Biochem **137**(1-2): 197-203.

Richardson, C. J., M. Broenstrup, D. C. Fingar, K. Julich, B. A. Ballif, S. Gygi and J. Blenis (2004). "SKAR is a specific target of S6 kinase 1 in cell growth control." Curr Biol **14**(17): 1540-1549.

Roberts, P. J. and C. J. Der (2007). "Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer." Oncogene **26**(22): 3291-3310.

Robida-Stubbs, S., K. Glover-Cutter, D. W. Lamming, M. Mizunuma, S. D. Narasimhan, E. Neumann-Haefelin, D. M. Sabatini and T. K. Blackwell (2012). "TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO." Cell Metab **15**(5): 713-724.

Robitaille, A. M., S. Christen, M. Shimobayashi, M. Cornu, L. L. Fava, S. Moes, C. Prescianotto-Baschong, U. Sauer, P. Jenoe and M. N. Hall (2013). "Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis." Science **339**(6125): 1320-1323.

Roczniak-Ferguson, A., C. S. Petit, F. Froehlich, S. Qian, J. Ky, B. Angarola, T. C. Walther and S. M. Ferguson (2012). "The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis." Sci Signal **5**(228): ra42.

Rosen, E. D. and O. A. MacDougald (2006). "Adipocyte differentiation from the inside out." Nat Rev Mol Cell Biol **7**(12): 885-896.

Rosen, E. D. and B. M. Spiegelman (2014). "What we talk about when we talk about fat." Cell **156**(1-2): 20-44.

Rosenwald, M., A. Perdikari, T. Rulicke and C. Wolfrum (2013). "Bi-directional interconversion of brite and white adipocytes." Nat Cell Biol **15**(6): 659-667.

Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst and S. H. Snyder (1994). "RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs." Cell **78**(1): 35-43.

Sancak, Y., L. Bar-Peled, R. Zoncu, A. L. Markhard, S. Nada and D. M. Sabatini (2010). "Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids." Cell **141**(2): 290-303.

Sancak, Y., T. R. Peterson, Y. D. Shaul, R. A. Lindquist, C. C. Thoreen, L. Bar-Peled and D. M. Sabatini (2008). "The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1." Science **320**(5882): 1496-1501.

Santalucia, T., M. Camps, A. Castello, P. Munoz, A. Nuel, X. Testar, M. Palacin and A. Zorzano (1992). "Developmental regulation of GLUT-1 (erythroid/Hep G2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue." Endocrinology **130**(2): 837-846.

Sarbassov, D. D., S. M. Ali, D. H. Kim, D. A. Guertin, R. R. Latek, H. Erdjument-Bromage, P. Tempst and D. M. Sabatini (2004). "Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton." Curr Biol **14**(14): 1296-1302.

Sarbassov, D. D., D. A. Guertin, S. M. Ali and D. M. Sabatini (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-1101.

Schenone, S., C. Brullo, F. Musumeci, M. Radi and M. Botta (2011). "ATP-competitive inhibitors of mTOR: an update." Curr Med Chem **18**(20): 2995-3014.

Seale, P., B. Bjork, W. Yang, S. Kajimura, S. Chin, S. Kuang, A. Scime, S. Devarakonda, H. M. Conroe, H. Erdjument-Bromage, P. Tempst, M. A. Rudnicki, D. R. Beier and B. M. Spiegelman (2008). "PRDM16 controls a brown fat/skeletal muscle switch." Nature **454**(7207): 961-967.

Seale, P., S. Kajimura, W. Yang, S. Chin, L. M. Rohas, M. Uldry, G. Tavernier, D. Langin and B. M. Spiegelman (2007). "Transcriptional control of brown fat determination by PRDM16." Cell Metab **6**(1): 38-54.

Sekulic, A., C. C. Hudson, J. L. Homme, P. Yin, D. M. Otterness, L. M. Karnitz and R. T. Abraham (2000). "A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells." Cancer Res **60**(13): 3504-3513.

Sengupta, S., T. R. Peterson, M. Laplante, S. Oh and D. M. Sabatini (2010). "mTORC1 controls fasting-induced ketogenesis and its modulation by ageing." Nature **468**(7327): 1100-1104.

Shah, O. J., Z. Wang and T. Hunter (2004). "Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies." Curr Biol **14**(18): 1650-1656.

Shahbazian, D., P. P. Roux, V. Mieulet, M. S. Cohen, B. Raught, J. Taunton, J. W. Hershey, J. Blenis, M. Pende and N. Sonenberg (2006). "The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity." EMBO J **25**(12): 2781-2791.

Shaw, R. J. and L. C. Cantley (2006). "Ras, PI(3)K and mTOR signalling controls tumour cell growth." Nature **441**(7092): 424-430.

Shibata, H., F. Perusse, A. Vallerand and L. J. Bukowiecki (1989). "Cold exposure reverses inhibitory effects of fasting on peripheral glucose uptake in rats." Am J Physiol **257**(1 Pt 2): R96-101.

Shimobayashi, M. and M. N. Hall (2014). "Making new contacts: the mTOR network in metabolism and signalling crosstalk." Nat Rev Mol Cell Biol **15**(3): 155-162.

Shinohara, Y., K. Yamamoto, K. Inoo, N. Yamazaki and H. Terada (1998). "Quantitative determinations of the steady state transcript levels of hexokinase isozymes and glucose transporter isoforms in normal rat tissues and the malignant tumor cell line AH130." Biochim Biophys Acta **1368**(1): 129-136.

Siljee, J. E., U. A. Unmehopa, A. Kalsbeek, D. F. Swaab, E. Fliers and A. Alkemade (2013). "Melanocortin 4 receptor distribution in the human hypothalamus." Eur J Endocrinol **168**(3): 361-369.

Simpson, L. and R. Parsons (2001). "PTEN: life as a tumor suppressor." Exp Cell Res **264**(1): 29-41.

Stahl, M., P. F. Dijkers, G. J. Kops, S. M. Lens, P. J. Coffer, B. M. Burgering and R. H. Medema (2002). "The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2." J Immunol **168**(10): 5024-5031.

Stan, R., M. M. McLaughlin, R. Cafferkey, R. K. Johnson, M. Rosenberg and G. P. Livi (1994). "Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue." J Biol Chem **269**(51): 32027-32030.

Suzuki, A., J. L. de la Pompa, V. Stambolic, A. J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, M. Fukumoto and T. W. Mak (1998). "High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice." Curr Biol **8**(21): 1169-1178.

Taniguchi, C. M., B. Emanuelli and C. R. Kahn (2006). "Critical nodes in signalling pathways: insights into insulin action." Nat Rev Mol Cell Biol **7**(2): 85-96.

Thoreen, C. C., L. Chantranupong, H. R. Keys, T. Wang, N. S. Gray and D. M. Sabatini (2012). "A unifying model for mTORC1-mediated regulation of mRNA translation." Nature **485**(7396): 109-113.

Tong, Q., C. P. Ye, J. E. Jones, J. K. Elmquist and B. B. Lowell (2008). "Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance." Nat Neurosci **11**(9): 998-1000.

Trayhurn, P. and J. H. Beattie (2001). "Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ." Proc Nutr Soc **60**(3): 329-339.

Trayhurn, P. and I. S. Wood (2004). "Adipokines: inflammation and the pleiotropic role of white adipose tissue." Br J Nutr **92**(3): 347-355.

Trotman, L. C., M. Niki, Z. A. Dotan, J. A. Koutcher, A. Di Cristofano, A. Xiao, A. S. Khoo, P. Roy-Burman, N. M. Greenberg, T. Van Dyke, C. Cordon-Cardo and P. P. Pandolfi (2003). "Pten dose dictates cancer progression in the prostate." PLoS Biol **1**(3): E59.

Ueno, M., J. B. Carvalheira, R. C. Tambascia, R. M. Bezerra, M. E. Amaral, E. M. Carneiro, F. Folli, K. G. Franchini and M. J. Saad (2005). "Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway." Diabetologia **48**(3): 506-518.

Um, S. H., F. Frigerio, M. Watanabe, F. Picard, M. Joaquin, M. Sticker, S. Fumagalli, P. R. Allegrini, S. C. Kozma, J. Auwerx and G. Thomas (2004). "Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity." Nature **431**(7005): 200-205.

Umemura, A., E. J. Park, K. Taniguchi, J. H. Lee, S. Shalapour, M. A. Valasek, M. Aghajan, H. Nakagawa, E. Seki, M. N. Hall and M. Karin (2014). "Liver damage, inflammation, and enhanced tumorigenesis after persistent mTORC1 inhibition." Cell Metab **20**(1): 133-144.

Vallerand, A. L., F. Perusse and L. J. Bukowiecki (1990). "Stimulatory effects of cold exposure and cold acclimation on glucose uptake in rat peripheral tissues." Am J Physiol **259**(5 Pt 2): R1043-1049.

van Marken Lichtenbelt, W. D., J. W. Vanhommerig, N. M. Smulders, J. M. Drossaerts, G. J. Kemerink, N. D. Bouvy, P. Schrauwen and G. J. Teule (2009). "Cold-activated brown adipose tissue in healthy men." N Engl J Med **360**(15): 1500-1508.

Varela, L. and T. L. Horvath (2012). "Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis." EMBO Rep **13**(12): 1079-1086.

Vellai, T., K. Takacs-Vellai, Y. Zhang, A. L. Kovacs, L. Orosz and F. Muller (2003). "Genetics: influence of TOR kinase on lifespan in C. elegans." Nature **426**(6967): 620.

Velloso, L. A., M. A. Torsoni and E. P. Araujo (2009). "Hypothalamic dysfunction in obesity." Rev Neurosci **20**(5-6): 441-449.

Vergnes, L., R. Chin, S. G. Young and K. Reue (2011). "Heart-type fatty acid-binding protein is essential for efficient brown adipose tissue fatty acid oxidation and cold tolerance." J Biol Chem **286**(1): 380-390.

Virtanen, K. A., M. E. Lidell, J. Orava, M. Heglind, R. Westergren, T. Niemi, M. Taittonen, J. Laine, N. J. Savisto, S. Enerback and P. Nuutila (2009). "Functional brown adipose tissue in healthy adults." N Engl J Med **360**(15): 1518-1525.

Wahlestedt, C. and D. J. Reis (1993). "Neuropeptide Y-related peptides and their receptors--are the receptors potential therapeutic drug targets?" Annu Rev Pharmacol Toxicol **33**: 309-352.

Wander, S. A., B. T. Hennessy and J. M. Slingerland (2011). "Next-generation mTOR inhibitors in clinical oncology: how pathway complexity informs therapeutic strategy." J Clin Invest **121**(4): 1231-1241.

Wang, S., J. Gao, Q. Lei, N. Rozengurt, C. Pritchard, J. Jiao, G. V. Thomas, G. Li, P. Roy-Burman, P. S. Nelson, X. Liu and H. Wu (2003). "Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer." Cancer Cell **4**(3): 209-221.

Wang, X., W. Li, M. Williams, N. Terada, D. R. Alessi and C. G. Proud (2001). "Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase." EMBO J **20**(16): 4370-4379.

Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel and A. W. Ferrante, Jr. (2003). "Obesity is associated with macrophage accumulation in adipose tissue." J Clin Invest **112**(12): 1796-1808.

Wenzel, H. J., L. S. Patel, C. A. Robbins, A. Emmi, R. S. Yeung and P. A. Schwartzkroin (2004). "Morphology of cerebral lesions in the Eker rat model of tuberous sclerosis." Acta Neuropathol **108**(2): 97-108.

Wernstedt Asterholm, I., C. Tao, T. S. Morley, Q. A. Wang, F. Delgado-Lopez, Z. V. Wang and P. E. Scherer (2014). "Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling." Cell Metab **20**(1): 103-118.

West, M. J., M. Stoneley and A. E. Willis (1998). "Translational induction of the c-myc oncogene via activation of the FRAP/TOR signalling pathway." Oncogene **17**(6): 769-780.

Wilding, J. P. (2002). "Neuropeptides and appetite control." Diabet Med **19**(8): 619-627.

Wilkinson, J. E., L. Burmeister, S. V. Brooks, C. C. Chan, S. Friedline, D. E. Harrison, J. F. Hejtmancik, N. Nadon, R. Strong, L. K. Wood, M. A. Woodward and R. A. Miller (2012). "Rapamycin slows aging in mice." Aging Cell **11**(4): 675-682.

Williams, L. M. (2012). "Hypothalamic dysfunction in obesity." Proc Nutr Soc **71**(4): 521-533.

Wilson, J. E. (2003). "Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function." J Exp Biol **206**(Pt 12): 2049-2057.

Wilson, K. F., W. J. Wu and R. A. Cerione (2000). "Cdc42 stimulates RNA splicing via the S6 kinase and a novel S6 kinase target, the nuclear cap-binding complex." J Biol Chem **275**(48): 37307-37310.

Woods, S. C. (2009). "The control of food intake: behavioral versus molecular perspectives." Cell Metab **9**(6): 489-498.

Wren, A. M., C. J. Small, C. R. Abbott, W. S. Dhillo, L. J. Seal, M. A. Cohen, R. L. Batterham, S. Taheri, S. A. Stanley, M. A. Ghatei and S. R. Bloom (2001). "Ghrelin causes hyperphagia and obesity in rats." Diabetes **50**(11): 2540-2547.

Wu, J., P. Bostrom, L. M. Sparks, L. Ye, J. H. Choi, A. H. Giang, M. Khandekar, K. A. Virtanen, P. Nuutila, G. Schaart, K. Huang, H. Tu, W. D. van Marken Lichtenbelt, J. Hoeks, S. Enerback, P. Schrauwen and B. M. Spiegelman (2012). "Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human." Cell **150**(2): 366-376.

Wu, Q., M. Kazantzis, H. Doege, A. M. Ortegon, B. Tsang, A. Falcon and A. Stahl (2006). "Fatty acid transport protein 1 is required for nonshivering thermogenesis in brown adipose tissue." Diabetes **55**(12): 3229-3237.

Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.

Xia, Y. and J. E. Wikberg (1997). "Postnatal expression of melanocortin-3 receptor in rat diencephalon and mesencephalon." Neuropharmacology **36**(2): 217-224.

Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia and H. Chen (2003). "Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance." J Clin Invest **112**(12): 1821-1830.

Yang, J. (2014). "Enhanced skeletal muscle for effective glucose homeostasis." Prog Mol Biol Transl Sci **121**: 133-163.

Yang, Q., K. Inoki, T. Ikenoue and K. L. Guan (2006). "Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity." Genes Dev **20**(20): 2820-2832.

Yang, S. B., A. C. Tien, G. Boddupalli, A. W. Xu, Y. N. Jan and L. Y. Jan (2012). "Rapamycin ameliorates age-dependent obesity associated with increased mTOR signaling in hypothalamic POMC neurons." Neuron **75**(3): 425-436.

Yaswen, L., N. Diehl, M. B. Brennan and U. Hochgeschwender (1999). "Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin." Nat Med **5**(9): 1066-1070.

Yecies, J. L., H. H. Zhang, S. Menon, S. Liu, D. Yecies, A. I. Lipovsky, C. Gorgun, D. J. Kwiatkowski, G. S. Hotamisligil, C. H. Lee and B. D. Manning (2011). "Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways." Cell Metab **14**(1): 21-32.

Yeo, G. S. and L. K. Heisler (2012). "Unraveling the brain regulation of appetite: lessons from genetics." Nat Neurosci **15**(10): 1343-1349.

Yeung, R. S., C. D. Katsetos and A. Klein-Szanto (1997). "Subependymal astrocytic hamartomas in the Eker rat model of tuberous sclerosis." Am J Pathol **151**(5): 1477-1486.

Yuan, M., E. Pino, L. Wu, M. Kacergis and A. A. Soukas (2012). "Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2." J Biol Chem **287**(35): 29579-29588.

Zha, J., H. Harada, E. Yang, J. Jockel and S. J. Korsmeyer (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L)." Cell **87**(4): 619-628.

Zhang, X., N. Tang, T. J. Hadden and A. K. Rishi (2011). "Akt, FoxO and regulation of apoptosis." Biochim Biophys Acta **1813**(11): 1978-1986.

Zinzalla, V., D. Stracka, W. Oppliger and M. N. Hall (2011). "Activation of mTORC2 by association with the ribosome." Cell **144**(5): 757-768.

Chapter 6: Appendix

6. Appendix

Rictor in Perivascular Adipose Tissue Controls Vascular Function by Regulating Inflammatory Molecule Expression

Indranil Bhattacharya, Katja Drägert, Verena Albert, Emmanuel Contassot, Marlen Damjanović, Asami Hagiwara, Lukas Zimmerli, Rok Humar, Michael N. Hall, Edouard J. Battegay, Elvira Haas

Objective—Perivascular adipose tissue (PVAT) wraps blood vessels and modulates vasoreactivity by secretion of vasoactive molecules. Mammalian target of rapamycin complex 2 (mTORC2) has been shown to control inflammation and is expressed in adipose tissue. In this study, we investigated whether adipose-specific deletion of rictor and thereby inactivation of mTORC2 in PVAT may modulate vascular function by increasing inflammation in PVAT.

Approach and Results—Rictor, an essential mTORC2 component, was deleted specifically in mouse adipose tissue ($rictor^{ad-/-}$). Phosphorylation of mTORC2 downstream target Akt at Serine 473 was reduced in PVAT from $rictor^{ad-/-}$ mice but unaffected in aortic tissue. Ex vivo functional analysis of thoracic aortae revealed increased contractions and impaired dilation in rings with PVAT from $rictor^{ad-/-}$ mice. Adipose rictor knockout increased gene expression and protein release of interleukin-6, macrophage inflammatory protein-1α, and tumor necrosis factor-α in PVAT as shown by quantitative real-time polymerase chain reaction and Bioplex analysis for the cytokines in the conditioned media, respectively. Moreover, gene and protein expression of inducible nitric oxide synthase was upregulated without affecting macrophage infiltration in PVAT from $rictor^{ad-/-}$ mice. Inhibition of inducible nitric oxide synthase normalized vascular reactivity in aortic rings from $rictor^{ad-/-}$ mice with no effect in $rictor^{fl/fl}$ mice. Interestingly, in perivascular and epididymal adipose depots, high-fat diet feeding induced downregulation of rictor gene expression.

Conclusions—Here, we identify mTORC2 as a critical regulator of PVAT-directed protection of normal vascular tone. Modulation of mTORC2 activity in adipose tissue may be a potential therapeutic approach for inflammation-related vascular damage. (***Arterioscler Thromb Vasc Biol*. 2013;33:2105-2111.)**

Key Words: adipose tissue ■ inflammation ■ nitric oxide synthase type II ■ perivascular adipose tissue ■ mammalian target of rapamycin complex 2 ■ thoracic aorta

Adipose tissue not only stores excess of energy as triacylglycerols but also secretes numerous growth factors, cytokines, and hormones that are involved in overall energy homeostasis and metabolism.[1] During obesity, adipose tissue mass increases and dysfunction occurs, favoring development of insulin resistance and cardiovascular disease.[1]

A crucial intracellular regulator of fatty acid metabolism and cell growth is the serine/threonine kinase mammalian target of rapamycin (mTOR).[2] mTOR exists in 2 multi-protein complexes, mTOR complex 1 (mTORC1) and mTORC2.[3] The latter complex is characterized by its essential regulatory protein rictor and integrates signals from growth factors to regulate cell survival or cytoskeleton organization.[4–7] mTORC2 phosphorylates AGC kinase family members, such as Akt at Serine 473.[8]

As full body knockout of rictor/mTORC2 is embryonically lethal,[9] diverse tissue-specific knockout models of rictor have been generated to characterize the physiological functions of rictor in the adult mouse.[4] Mice with specific ablation of rictor in adipose tissue have higher lean mass because of increased levels of insulin and insulin-like growth factor-1, display enhanced glucose metabolism, and are insulin resistant.[10,11]

Among the various and distinct adipose depots, perivascular adipose tissue (PVAT) not only serves as a structural support for most arteries but also secretes molecules to actively modulate vascular function.[12,13] In 1991, Soltis and Cassis[14] showed for the first time that PVAT has an anticontractile function. In 2002, Löhn et al[15] reported PVAT to release a transferable vasoactive factor that acts on vascular smooth muscle cells via ATP-dependent K^+ channels to mediate vasorelaxation. Since then, the list of dilatory or anticontractile molecules released from PVAT has expanded and now includes adipokines (eg, adiponectin),[16] reactive oxygen species (eg, hydrogen peroxide),[17] and others (eg, methyl palmitate,[18] hydrogen sulfide,[19] and angiotensin 1–7[20]). In 2005, Yudkin et al[21] proposed that obesity and its adverse effects cause PVAT to inflame and

Received on: December 13, 2012; final version accepted on: July 1, 2013.
From the Research Unit, Division of Internal Medicine (I.B., K.D., M.D., L.Z., R.H., E.J.B., E.H.) and Department of Dermatology (E.C.), University Hospital of Zurich, Zurich, Switzerland; Growth and Development Biozentrum, University of Basel, Basel, Switzerland (V.A., A.H., M.N.H.); and Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland (R.H., E.J.B.).
The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.301001/-/DC1.
Correspondence to Elvira Haas, PhD, Research Unit, Division of Internal Medicine, University Hospital Zurich, Gloriastr. 30, CH-8091 Zurich, Switzerland. E-mail Elvira.haas@usz.ch

to secrete tumor necrosis factor-α (TNFα) and interleukin-6 (IL6), which resulted in loss of PVAT's anticontractile function. In rat mesenteric arteries, these inflammatory cytokines impaired the anticontractile activity of healthy PVAT,[22] and in mouse femoral arteries, endovascular injury upregulated inflammation in PVAT.[23]

Thus, the progression of the inflammatory reaction in PVAT seems to be crucial in altering PVAT's vasoactive properties; however, intracellular mechanisms that might control these reactions are not known. Recently, mTORC2-deficient mouse embryonic fibroblasts and rictor knockdown of dendritic cells exhibited a hyperinflammatory phenotype after lipopolysaccharide stimulation.[24] In the present study, we have, therefore, tested the hypothesis that deletion of rictor in adipose tissue, and thereby inactivation of mTORC2 in PVAT, may modulate vascular function by increasing inflammation in PVAT.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Adipose-Specific Deletion of Rictor Attenuates mTORC2 Signaling in Aortic PVAT

To analyze the role of mTORC2 in PVAT for vascular function, we used adipose cell-specific rictor knockout (rictor$^{ad-/-}$) and corresponding control (rictor$^{fl/fl}$) mice, which were described previously.[10] Adipose-specific rictor knockout was confirmed by significant reduction of *rictor* gene expression in epididymal fat (EFAT) from rictor$^{ad-/-}$ mice (Figure I in the online-only Data Supplement). Next, *rictor* gene expression in aorta (tissue containing mainly smooth muscle and endothelial cells) and in PVAT surrounding the thoracic aorta (referred henceforth as PVAT) was examined. The expression of *rictor* was significantly reduced in the PVAT but remained unchanged in the aorta from rictor$^{ad-/-}$ mice (Figure 1A). To evaluate mTORC2 activity, aortic rings with intact PVAT were stimulated with insulin. Subsequently, PVAT and aorta were separated and analyzed for Akt Ser473 phosphorylation, a known TORC2 substrate.[8] PVAT from rictor$^{ad-/-}$ mice exhibited reduced Akt Ser473 phosphorylation after insulin stimulation indicating impaired insulin signaling (Figure 1B and 1C). In the aorta, insulin-induced Akt Ser473 phosphorylation remained unchanged in the groups (Figure 1B and 1C). No difference was detected in insulin-stimulated phosphorylation of extracellular signal-regulated protein kinase 1 and 2 between the mice groups in PVAT and aorta (Figure 1B).

Increased Aortic Contraction and Impaired Endothelium-Dependent Relaxation in Rictor$^{ad-/-}$ Mice in the Presence of PVAT

PVAT surrounding the arteries influences vascular function.[12,13] To investigate whether adipose-specific rictor deletion modulate aortic contractions, aortic rings with or without PVAT were used. To test the contractile capacity of the smooth muscle cell layer by direct depolarization, all rings were initially treated with 100 mmol/L potassium chloride (KCl). In all rings, similar contractions independent of rictor and PVAT were observed (data not shown). Next, we investigated the

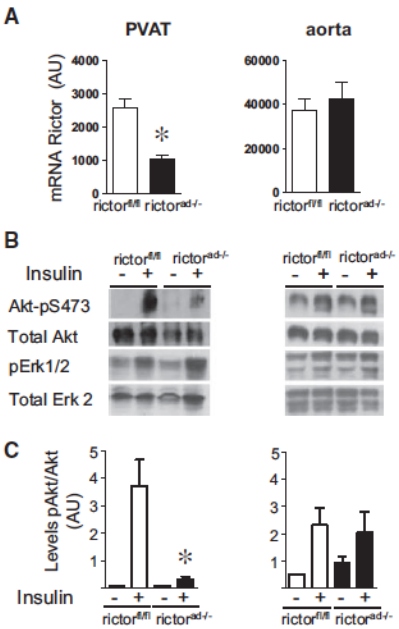


Figure 1. Adipose cell-specific deletion of rictor results in decreased expression of rictor and reduced Akt phosphorylation in perivascular adipose tissue (PVAT). **A**, Steady-state mRNA expression levels of *rictor* in PVAT (n=9/10) and aortic tissue (n=4) were determined in rictor$^{fl/fl}$ and rictor$^{ad-/-}$ mice using quantitative real-time polymerase chain reaction. **B**, Immunoblot analysis of insulin-stimulated phosphorylation of Akt at Ser473 and extracellular signal-regulated protein kinase 1 and 2 (Erk1/2) in PVAT and aorta from rictor$^{fl/fl}$ and rictor$^{ad-/-}$ mice. Total Akt and Erk2 were used as control for protein loading. One representative blot of 3 is shown. **C**, Quantification of pAkt in PVAT and aorta from rictor$^{fl/fl}$ and rictor$^{ad-/-}$ mice (n=3). Densitometric analysis was first normalized to total Akt and then to untreated rictor$^{fl/fl}$. All data represent mean±SEM. *P<0.05 vs rictor$^{fl/fl}$.

contribution of rictor in PVAT to receptor-dependent vascular contraction. Aortic rings with or without PVAT were exposed to vasoconstrictor phenylephrine (PE) in a concentration-dependent manner. Aortic rings with PVAT from rictor$^{ad-/-}$ mice displayed 2.1-fold higher maximal contractions as compared with rings from rictor$^{fl/fl}$ mice after treatment with PE (rictor$^{ad-/-}$: 43.2±6.3% versus rictor$^{fl/fl}$: 20.1±3.8% at 3×10^{-6} mol/L; Figure 2A). With the removal of PVAT, contractions to PE increased overall and maximal contractions were not significantly different between both groups of mice (rictor$^{ad-/-}$: 70.0±8.6% versus rictor$^{fl/fl}$: 81.0±10.1% at 3×10^{-6} mol/L; Figure 2B). To determine whether vascular relaxation was also affected, we analyzed acetylcholine-induced endothelium-dependent relaxation. Acetylcholine-induced relaxation in aortic rings with PVAT was impaired in rictor$^{ad-/-}$ mice (Figure 2C). This impairment was also confirmed by the pD_2 values, which suggests a delay in relaxation in rictor$^{ad-/-}$ aortic rings with PVAT (rictor$^{ad-/-}$: 6.2±0.2 mol/L versus rictor$^{fl/fl}$: 7.0±0.1 mol/L). On removal of PVAT, the differences were lost and the aortic rings relaxed normally (Figure 2D). As we found differences in contraction and dilation to

receptor-dependent agonists in rings with PVAT, we further characterized contraction to KCl. Concentration-dependent (10–100 mmol/L) KCl-induced contractions in aortic rings with PVAT were similar between both groups of mice (Figure II in the online-only Data Supplement).

Hypothetically, increased aortic contraction with PVAT in rictor$^{ad-/-}$ mice might be because of reduced nitric oxide (NO) availability. To investigate this, we used N^G-nitro-L-arginine methyl ester (L-NAME) which preferentially inhibits endothelial nitric oxide synthase (eNOS).[25] In the presence of L-NAME (300×10^{-6} mol/L), the contractile responses to PE increased in rings with PVAT from both mice groups (Figure 2E). Nonetheless, PE-induced contractions were higher in aortic rings with PVAT from rictor$^{ad-/-}$ mice (1.5-fold; rictor$^{ad-/-}$: 86.36±8.17% versus rictor$^{fl/fl}$: 58.78±6.71% at 3×10^{-6} mol/L; Figure 2E).

To define the specificity of L-NAME for the different NO synthases, we used RAW264.7 cell line which exclusively expresses inducible NOS (iNOS/NOS2).[26] NO measurements after lipopolysaccharide stimulation revealed that L-NAME at 300×10^{-6} mol/L also partially inhibited iNOS-induced NO production (≈40%; Figure III in the online-only Data Supplement). These results suggest that L-NAME is a non-selective NOS inhibitor. Expectedly, iNOS inhibitor 1400 W (10^{-6} mol/L), a highly specific and selective inhibitor,[27] inhibited lipopolysaccharide-induced NO production to baseline levels (Figure III in the online-only Data Supplement).

In addition to NO, endothelium also releases other dilatory factors. Hence, experiments were performed with endothelium-denuded aortic rings with PVAT. Aortic contractions to PE or prostaglandin $F_{2\alpha}$ were significantly higher in rictor$^{ad-/-}$ mice compared with rictor$^{fl/fl}$ mice (Figure 2F and 2G). Taken together, these results suggest that the anticontractile effect mediated by rictor is for the most part endothelium-independent.

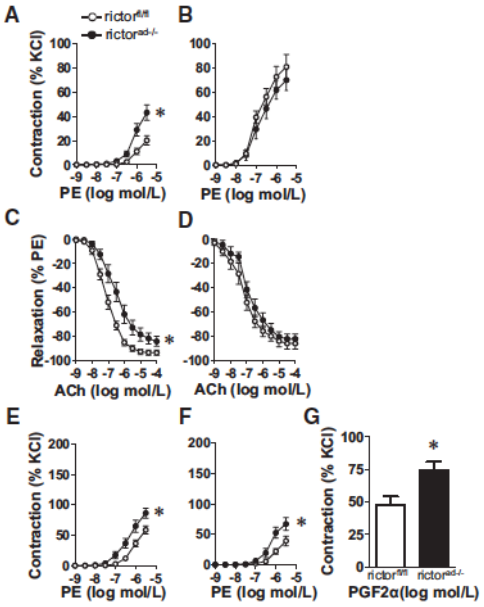


Figure 2. Rictor controls anticontractility in perivascular adipose tissue (PVAT). Aortic rings from rictor$^{fl/fl}$ and rictor$^{ad-/-}$ mice were used. Rings with PVAT (**A**) or without PVAT (**B**) were contracted with phenylephrine (PE; 10^{-9} to 3×10^{-6} mol/L) in a concentration-dependent manner (n=10–13). Rings with PVAT (**C**) or without PVAT (**D**) were precontracted with PE and treated with acetylcholine (ACh; 10^{-9} to 10^{-4} mol/L) in a concentration-dependent manner (n=7–13). **E**, Aortic rings with PVAT were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME) (300×10^{-6} mol/L) and then contracted with PE in a concentration-dependent manner (n=5–13). **F** and **G**, Aortic rings with PVAT but without endothelium were treated in a concentration-dependent manner with PE or with 3×10^{-6} mol/L of prostaglandin $F_{2\alpha}$ (n=6–9). All values are mean±SEM. *P<0.05 vs rictor$^{fl/fl}$.

Loss of Rictor in Adipose Cells of PVAT Results in Upregulation of Inflammatory Mediators

To determine how loss of rictor in adipose cells of PVAT increases aortic contractions and impairs relaxation, we screened genes which are expressed in and secreted by PVAT. These included adipokines,[28] reactive oxygen species,[17,29] and inflammatory molecules.[30] Among the adipokines, gene expression levels of *adiponectin* (*adipoq*) and *lipocalin* (*lnc2*) were not affected in the PVAT from rictor$^{ad-/-}$ mice (Figure 3A). However, the expression level of *resistin* (*retn*) was significantly reduced. Gene expression of NADPH oxidase subunits *nox2*, *nox4*, *p22phox*, and *p67phox* and adipocyte markers (*Pparg* and *Fabp4*) were not affected. Interestingly, the expression of inflammatory genes, such as

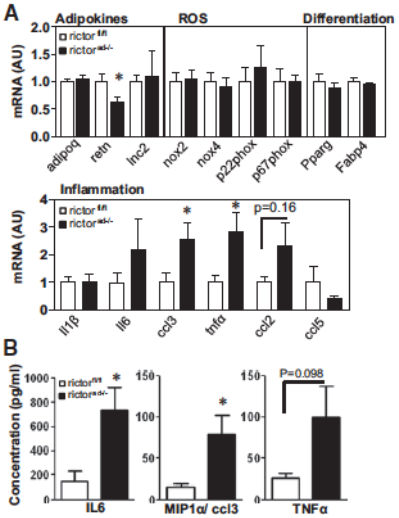


Figure 3. Adipocyte-specific deletion of rictor increases expression of inflammatory molecules in perivascular adipose tissue (PVAT). **A**, Steady-state mRNA expression levels of adipokines, reactive oxygen species (ROS), adipocyte differentiation, and inflammatory molecules as determined by quantitative real-time polymerase chain reaction. Results are normalized to the levels in PVAT from rictor$^{fl/fl}$ mice (n=4–8). **B**, Protein concentrations of inflammatory cytokines released by PVAT were determined in conditioned media using Bioplex system (n=4). All values represent mean±SEM, *P<0.05 vs rictor$^{fl/fl}$. IL indicates interleukin-6; MIP-1α, macrophage inflammatory protein-1α; and TNFα, tumor necrosis factor-α.

chemokine (C-C-motif) ligand 3 (Ccl3)/macrophage inflammatory protein 1α (Mip1α), and *Tnfα*, was increased in the PVAT from rictor$^{ad-/-}$ mice (Figure 3A).

Protein analysis of conditioned media revealed increased release of IL6 (5-fold), Mip1α (5-fold), and Tnfα (4-fold) from PVAT of rictor$^{ad-/-}$ mice (Figure 3B).

Increased iNOS Expression but Unchanged Leukocytes Levels in PVAT from Rictor$^{ad-/-}$ Mice

To investigate whether the augmented expression of inflammatory molecules was associated with elevated macrophage infiltration in PVAT, we determined steady-state mRNA expression levels of macrophage-associated genes *emr1* (*F4/80*), *CD163*, and *Nos2/iNos*. Expression of *F4/80* and *CD163* was similar between groups, whereas *iNos* expression increased significantly in PVAT from rictor$^{ad-/-}$ mice (Figure 4A). To validate this, we examined the expression of iNOS in cross sections of thoracic aorta with PVAT. Indeed, iNOS expression was higher in the PVAT from rictor$^{ad-/-}$ mice (Figure 4B). The uniform expression of iNOS across the PVAT suggests that adipocytes themselves and not macrophages are the predominant cells expressing iNOS. Moreover, iNOS staining in aortic medial layer was hardly visible.

Using flow cytometry, the infiltration of inflammatory cells was quantified. The percentages of total leukocytes and macrophages in the stromal vascular fractions were similar between groups in PVAT (Figure 4C). In EFAT, no differences in leukocyte and macrophage percentages in stromal vascular fractions were detected, but overall percentages of leukocytes and macrophages were increased ≈100% when compared with PVAT (Figure 4C).

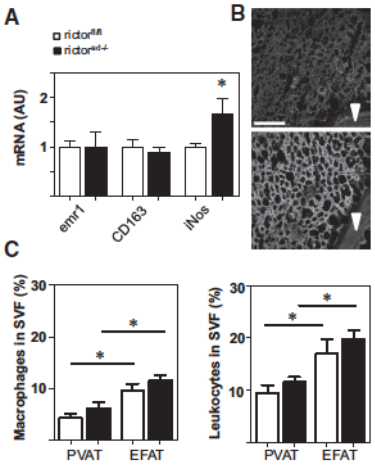


Figure 4. Elevated inducible nitric oxide synthase (iNOS) expression in perivascular adipose tissue (PVAT) from rictor$^{ad-/-}$ mice is independent of macrophage infiltration. **A**, Steady-state mRNA expression levels of *emr1* (*F4/80*), *CD163*, and *Nos2/iNos* were determined by quantitative real-time polymerase chain reaction. Results are normalized to the levels in PVAT from rictor$^{fl/fl}$ mice (n=8–9), *P<0.05 vs rictor$^{fl/fl}$. **B**, Immunofluorescence staining of iNOS in cross sections of thoracic aorta with PVAT from rictor$^{fl/fl}$ (**top**) and rictor$^{ad-/-}$ (**bottom**) mice. Arrowheads indicate aortic medial layer. Scale bar, 100 μm. Images are representative of n≥3 mice. **C**, Percentages of macrophages (F4/80 and CD45.2 positive cells) and leukocytes (CD45.2 positive cells) in stromal vascular fraction (SVF) from PVAT and epididymal fat (EFAT) using flow cytometry (n=4–5). All values represent mean±SEM. *P<0.05.

Inhibition of iNOS Restores Anticontractile Effect of PVAT

We analyzed the effect of TNFα on PE-induced contractions, as this proinflammatory cytokine has been shown to counteract the anticontractile activity of PVAT.[22] In aortic rings with PVAT from rictor$^{fl/fl}$ mice, contractions to PE (10^{-6} mol/L) increased in the presence TNFα but not to a similar level as contractions in the rings from rictor$^{ad-/-}$ mice, which were significantly higher compared with untreated rings from rictor$^{fl/fl}$ mice (Figure IV in the online-only Data Supplement). TNFα stimulated iNos expression in 3T3-L1 adipocytes (Figure V in the online-only Data Supplement) as reported previously.[31] To confirm our observation that iNOS is expressed mainly in PVAT (Figure 4B, only background staining in the medial layer of the aorta), we analyzed gene expression of *iNos* in aortic tissue and PVAT. Indeed, expression of *iNos* was only detected in PVAT but not in the aorta of rictor$^{ad-/-}$ mice using quantitative real-time polymerase chain reaction (cycle threshold value in PVAT: 29; aorta: no amplification). Thus, we investigated the possible role of iNOS for vascular function using iNOS inhibitor 1400 W.[27] In aortic rings with PVAT, contractions in rictor$^{ad-/-}$ mice were reduced in the presence of 1400 W (Figure 5A), reaching similar levels detected in rictor$^{fl/fl}$ mice (Figure 5B). In addition, treatment with 1400 W improved the endothelium-dependent relaxation in aortic rings with PVAT from rictor$^{ad-/-}$ mice (Figure 5C) to comparable levels observed in control rings (Figure 5D). Thus, the altered function in aortic rings with PVAT in rictor$^{ad-/-}$ mice was normalized after inhibition of iNOS. Taken together, our

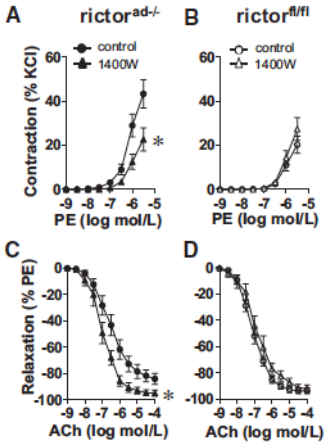


Figure 5. Inhibition of inducible nitric oxide synthase (iNOS) in perivascular adipose tissue (PVAT) normalizes vascular reactivity in rictor$^{ad-/-}$ mice. **A** and **B**, Aortic rings with PVAT from rictor$^{ad-/-}$ and rictor$^{fl/fl}$ mice were contracted with phenylephrine (PE) in the presence or absence of iNOS inhibitor 1400 W (10^{-6} mol/L). rictor$^{ad-/-}$ pD2: 5.4±0.2 mol/L vs rictor$^{ad-/-}$+1400 W pD2: 4.8±0.2 mol/L (n=11–13). *P<0.05 vs rictor$^{ad-/-}$ by pD2. **C** and **D**, Precontracted aortic rings with PVAT were relaxed using acetylcholine (ACh) in the presence or absence of 1400 W (n=7–13). All values are mean±SEM. *P<0.05 vs rictor$^{ad-/-}$.

findings suggest that increased levels of TNFα in concert with iNOS-derived NO metabolites and possibly other inflammation-associated molecules counteract the intrinsic anticontractile function of PVAT from rictor$^{ad-/-}$ mice.

Downregulation of Rictor Expression in Adipose Tissue After High-Fat Diet Feeding

Because depletion of rictor in adipose tissue is associated with increased expression of inflammatory genes and proteins in PVAT, we assessed the possible physiological relevance of rictor expressional regulation after high-fat diet (HFD) feeding, a model of experimental obesity and chronic low-grade inflammatory disease.[32]

We analyzed *rictor* gene expression levels in PVAT and EFAT in control mice fed for 10 to 12 weeks with a HFD and with control diet. Steady-state mRNA expression levels of *rictor* were significantly downregulated in both types of adipose tissue after HFD (>30%; Figure 6A and 6B, left panels). Concomitantly, *iNos* expression was significantly increased in PVAT of these HFD fed mice, whereas in EFAT only an upward trend was observed (P=0.16). In June 2013, a search in published micro array data sets in NCBI Gene Expression Omnibus database[33] revealed that *rictor* mRNA expression levels were also significantly lower in white adipose tissue from C57Bl6/J mice after 8 weeks of HFD feeding (accession number GSE30247; Figure VI in the online-only Data Supplement), whereas inflammatory genes were upregulated.[34]

Discussion

In the present study, we describe a hitherto unknown function of the serine and threonine kinase complex mTORC2 in controlling the anticontractile activity of PVAT primarily by regulating inflammation in the adipocytes. Downregulation of rictor, an essential mTORC2 component, in adipose tissue increased gene expression of *Tnfα*, *Mip1α*, and *iNos* in PVAT and, concurrently increased the secretion of IL6, MIP1α, and TNFα. Moreover, inhibition of iNOS restored the anticontractile activity of PVAT lacking rictor. Importantly, we demonstrate that in a model of low-grade chronic inflammation, such as HFD-induced obesity, gene expression of *rictor* in PVAT is downregulated, whereas that of *iNos* is upregulated.

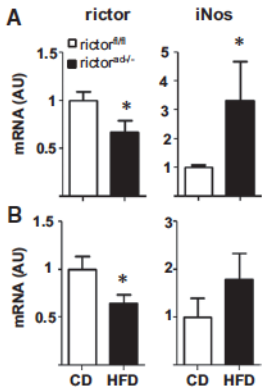


Figure 6. Differential effects of high-fat diet (HFD) feeding on expression of rictor and inducible nitric oxide synthase (iNos) in fat depots. Steady-state mRNA expression levels of rictor and iNos in PVAT (**A**) and epididymal fat (**B**) of mice fed with control diet (CD; n=5–9) or HFD (n=6–9). Results are normalized to the levels from mice fed CD. Values are mean±SEM. *P<0.05 vs CD.

Studies addressing PVAT's contribution to vascular functions have largely focused on identifying molecules released by PVAT in normal and in pathophysiological conditions, such as obesity or hypertension.[13,30] Signaling enzymes in PVAT that control the expression and secretion of these secretory molecules are not yet known. To the best of our knowledge, this is the first study to show the contribution of an intracellular signaling molecule (rictor) in adipocytes of PVAT in regulating the anticontractile activity.

Jabs et al[35] have shown that continuous treatment with the mTOR inhibitor rapamycin, also known as sirolimus, markedly reduced vasorelaxation to acetylcholine in rat aortae devoid of PVAT by inducing endothelial dysfunction associated with reduced NO availability. Possible underlying mechanism may be the direct inhibition of mTORC1. In endothelial cells, however, also mTORC2 assembly after long-term rapamycin treatment is compromised,[36] impairing downstream signaling from Akt to endothelial NOS.[37,38] In our study, depletion of rictor from adipose cells in PVAT increased aortic contractions in response to receptor-dependent vasoconstrictors and impaired vasorelaxation. On removing PVAT, the differences in contraction and dilation between the mice groups vanished, supporting the current model stating that PVAT-released molecules directly mediate vasoactive effects.[13]

PVAT can exert its anticontractile effects via endothelium-dependent or endothelium-independent mechanisms.[17,20] The endothelium-dependent effect involves production of NO by endothelial NOS, which is required to maintain vascular tone, regulate platelet aggregation and leukocyte adhesion.[39] In our model, the anticontractile function of PVAT was still compromised after endothelium denudation in rictor$^{ad-/-}$ mice compared with controls. Thus, endothelium-derived vasodilators do not play a major role in anticontractile effects mediated by mTORC2 in PVAT. Inherent contractile responsiveness of vascular smooth muscle cells in the aortic rings was also preserved, despite the loss of rictor in PVAT because KCl-induced contractions were identical. This suggests that anticontractile effects mediated via mTORC2 in PVAT are most likely caused by modulation of the secretory proteins affecting adjacent vascular cells without changing their inherent functionality.

Increased inflammation and oxidative stress in PVAT have been linked to promote vasoconstriction and endothelial dysfunction.[40,41] In this regard, we screened the expression of reactive oxygen species, generating NADPH oxidase subunits and inflammatory cytokines. In PVAT lacking rictor, expression levels of iNos and inflammatory cytokines, such as IL6, MIP1α, and TNFα, were increased. Thus, in murine PVAT, decreased mTORC2 activity initiated a cascade of inflammatory pathways.

Vascular expression of iNOS occurs during pathological changes in the vascular wall such as atherosclerosis development, hypertension, and vascular injury.[42] These pathologies are often associated with inflammatory processes which induce expression of iNOS in the vascular wall. Healthy blood vessels do not express iNOS.[43] Consistent with this finding,

we did not detect iNOS expression in thoracic arterial tissue, that is, endothelium plus vascular smooth muscle cells, without PVAT. Tian et al[44] have demonstrated that inhibition of iNOS protects endothelium/NO-dependent vasodilation of thoracic aorta in aged rats. Vice versa, overexpression of iNOS using gene transfer resulted in impaired NO-dependent vasorelaxation in carotid arteries.[45]

In our work, partial inhibition of iNOS by 40% using nonselective NOS inhibitor L-NAME was not sufficient to adjust PE-mediated contractions between groups, whereas specific and complete inhibition of iNOS using 1400 W completely restored vasodilation and anticontractile activity in rings with PVAT lacking adipose-specific rictor. We propose that PVAT from rictor$^{ad-/-}$ mice has preserved vasodilator effects which are compromised by the action of upregulated procontractile mediators such as iNOS-derived NO metabolites and proinflammatory cytokines. As a consequence, the procontractile molecules counteract but cannot abolish the overall anticontractile activity exerted, which is ultimately lost only after removal of PVAT in rings from rictor$^{ad-/-}$ mice.

The marked reduction in insulin-stimulated Akt phosphorylation in PVAT of rictor$^{ad-/-}$ mice may not only be caused by reduced mTORC2 to Akt signaling but may also be attributed to insulin resistance in these mice,[10,11] resulting in impaired insulin signaling already at the level of the insulin receptor substrates.[46] Brown et al[24] have demonstrated that expression of a constitutively active Akt variant normalizes the hyperinflammatory phenotype in rictor-deficient cells by restoring mTORC2 downstream signaling. Thus, these increased inflammatory expression profiles observed in our study may possibly be caused by defective Akt signaling in PVAT lacking rictor.

Infiltration of macrophages and T cells in PVAT has been reported in mice fed with HFD.[28,30] In contrast, thoracic PVAT has also been reported to be fairly resistant to macrophage infiltration, to obesity-induced inflammation, and is nearly identical to interscapular brown adipose tissue.[47] In line with these findings, we found in our study about half as many leukocytes and macrophages in vascular stromal fractions of PVAT as compared with those of EFAT. These observations argue against an intrinsically proinflammatory depot in thoracic PVAT. Moreover, there was no difference in macrophage presence in PVAT between rictor$^{fl/fl}$ and rictor$^{ad-/-}$ mice. Thus, we suggest that ablation of rictor in adipocytes initiates expressional changes to upregulate release of inflammatory molecules in the adipocyte itself which counteract PVAT's anticontractile function.

The hyperinflammatory phenotype in PVAT caused by rictor deletion in adipocytes resembles obesity-induced changes.[22,48,49] Increased expression of inflammatory cytokines, such as TNFα and IL6 in PVAT of obese patients, has been implicated to reduce its anticontractile activity.[22] In line, a previous study by Meijer et al[50] showed that inhibition of inflammation restored anticontractile function of microvascular PVAT in a genetic model of obesity.

Intriguingly in our study, experimental obesity in mice induced via HFD feeding per se downregulated expression of *rictor* in thoracic PVAT, whereas *iNos* expression was upregulated. Considering the complexity and the interplay of different molecules and cells involved in obesity-induced adipose tissue inflammation, it was beyond the scope of the present study to elucidate mechanisms underlying the downregulation of rictor expression in this context. We speculate that several inflammatory molecules, including TNFα, induce a series of signaling cascades in perivascular adipocytes resulting in downregulation of rictor expression. Furthermore, reduced mTORC2 assembly and signaling, in turn, might further advance the ongoing inflammatory response. Thus, mTORC2 signaling is highly likely to participate in the complex regulation of obesity-associated adipose tissue inflammation.

In conclusion, the present findings assign a decisive function to rictor in PVAT in protecting arteries from inflammatory stress and in controlling normal vascular function. Stabilizing expression of rictor in adipose tissue during obesity and other metabolic rearrangements might, therefore, embody a novel treatment approach to combat inflammation-associated vascular damage.

Acknowledgments

We thank Dr Chafik Ghayor for the help with RAW264.7 cells experiments. We are grateful to Ana Perez-Dominguez and Emerita Ammann Meier for their expert technical assistance.

Sources of Funding

This work was supported by funding from University of Zurich, University Hospital of Zurich, and Swiss National Science Foundation.

Disclosures

None.

References

1. Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur Heart J*. 2008;29:2959–2971.
2. Soliman GA. The integral role of mTOR in lipid metabolism. *Cell Cycle*. 2011;10:861–862.
3. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*. 2002;10:457–468.
4. Oh WJ, Jacinto E. mTOR complex 2 signaling and functions. *Cell Cycle*. 2011;10:2305–2316.
5. Cybulski N, Hall MN. TOR complex 2: a signaling pathway of its own. *Trends Biochem Sci*. 2009;34:620–627.
6. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*. 2004;14:1296–1302.
7. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*. 2004;6:1122–1128.
8. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005;307:1098–1101.
9. Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA. Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Dev Cell*. 2006;11:583–589.
10. Cybulski N, Polak P, Auwerx J, Rüegg MA, Hall MN. mTOR complex 2 in adipose tissue negatively controls whole-body growth. *Proc Natl Acad Sci U S A*. 2009;106:9902–9907.
11. Kumar A, Lawrence JC Jr, Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA, Harris TE. Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism. *Diabetes*. 2010;59:1397–1406.
12. Aghamohammadzadeh R, Withers S, Lynch F, Greenstein A, Malik R, Heagerty A. Perivascular adipose tissue from human systemic and coronary vessels: the emergence of a new pharmacotherapeutic target. *Br J Pharmacol*. 2012;165:670–682.
13. Szasz T, Webb RC. Perivascular adipose tissue: more than just structural support. *Clin Sci (Lond)*. 2012;122:1–12.
14. Soltis EE, Cassis LA. Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A*. 1991;13:277–296.

15. Löhn M, Dubrovska G, Lauterbach B, Luft FC, Gollasch M, Sharma AM. Periadventitial fat releases a vascular relaxing factor. *FASEB J*. 2002;16:1057–1063.
16. Fésüs G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft FC, Gollasch M. Adiponectin is a novel humoral vasodilator. *Cardiovasc Res*. 2007;75:719–727.
17. Gao YJ, Lu C, Su LY, Sharma AM, Lee RM. Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. *Br J Pharmacol*. 2007;151:323–331.
18. Lee YC, Chang HH, Chiang CL, Liu CH, Yeh JI, Chen MF, Chen PY, Kuo JS, Lee TJ. Role of perivascular adipose tissue-derived methyl palmitate in vascular tone regulation and pathogenesis of hypertension. *Circulation*. 2011;124:1160–1171.
19. Köhn C, Schleifenbaum J, Szijártó IA, Markó L, Dubrovska G, Huang Y, Gollasch M. Differential effects of cystathionine-γ-lyase-dependent vasodilatory H2S in periadventitial vasoregulation of rat and mouse aortas. *PLoS One*. 2012;7:e41951.
20. Lee RM, Lu C, Su LY, Gao YJ. Endothelium-dependent relaxation factor released by perivascular adipose tissue. *J Hypertens*. 2009;27:782–790.
21. Yudkin JS, Eringa E, Stehouwer CD. "Vasocrine" signalling from perivascular fat: a mechanism linking insulin resistance to vascular disease. *Lancet*. 2005;365:1817–1820.
22. Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates AP, Pemberton PW, Malik RA, Heagerty AM. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation*. 2009;119:1661–1670.
23. Takaoka M, Suzuki H, Shioda S, Sekikawa K, Saito Y, Nagai R, Sata M. Endovascular injury induces rapid phenotypic changes in perivascular adipose tissue. *Arterioscler Thromb Vasc Biol*. 2010;30:1576–1582.
24. Brown J, Wang H, Suttles J, Graves DT, Martin M. Mammalian target of rapamycin complex 2 (mTORC2) negatively regulates Toll-like receptor 4-mediated inflammatory response via FoxO1. *J Biol Chem*. 2011;286:44295–44305.
25. Luo Z, Fujio Y, Kureishi Y, Rudic RD, Daumerie G, Fulton D, Sessa WC, Walsh K. Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity in vivo. *J Clin Invest*. 2000;106:493–499.
26. Naureckiene S, Edris W, Ajit SK, Katz AH, Sreekumar K, Rogers KE, Kennedy JD, Jones PG. Use of a murine cell line for identification of human nitric oxide synthase inhibitors. *J Pharmacol Toxicol Methods*. 2007;55:303–313.
27. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, Knowles RG. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and in vivo. *J Biol Chem*. 1997;272:4959–4963.
28. Takaoka M, Nagata D, Kihara S, Shimomura I, Kimura Y, Tabata Y, Saito Y, Nagai R, Sata M. Periadventitial adipose tissue plays a critical role in vascular remodeling. *Circ Res*. 2009;105:906–911.
29. Gao YJ, Takemori K, Su LY, An WS, Lu C, Sharma AM, Lee RM. Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. *Cardiovasc Res*. 2006;71:363–373.
30. Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G, Rothenberg FG, Neltner B, Romig-Martin SA, Dickson EW, Rudich S, Weintraub NL. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. *Circ Res*. 2009;104:541–549.
31. Juan CC, Lien CC, Chang CL, Huang YH, Ho LT. Involvement of iNOS and NO in TNF-alpha-downregulated resistin gene expression in 3T3-L1 adipocytes. *Obesity (Silver Spring)*. 2008;16:1219–1225.
32. Hassan M, Latif N, Yacoub M. Adipose tissue: friend or foe? *Nat Rev Cardiol*. 2012;9:689–702.
33. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30:207–210.
34. Chalkiadaki A, Guarente L. High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction. *Cell Metab*. 2012;16:180–188.
35. Jabs A, Göbel S, Wenzel P, Kleschyov AL, Hortmann M, Oelze M, Daiber A, Münzel T. Sirolimus-induced vascular dysfunction. Increased mitochondrial and nicotinamide adenosine dinucleotide phosphate oxidase-dependent superoxide production and decreased vascular nitric oxide formation. *J Am Coll Cardiol*. 2008;51:2130–2138.
36. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*. 2006;22:159–168.
37. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605.
38. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601.
39. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A*. 1991;88:4651–4655.
40. Withers SB, Agabiti-Rosei C, Livingstone DM, Little MC, Aslam R, Malik RA, Heagerty AM. Macrophage activation is responsible for loss of anticontractile function in inflamed perivascular fat. *Arterioscler Thromb Vasc Biol*. 2011;31:908–913.
41. Ketonen J, Shi J, Martonen E, Mervaala E. Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57Bl/6 mice. *Circ J*. 2010;74:1479–1487.
42. Ginnan R, Guikema BJ, Halligan KE, Singer HA, Jourd'heuil D. Regulation of smooth muscle by inducible nitric oxide synthase and NADPH oxidase in vascular proliferative diseases. *Free Radic Biol Med*. 2008;44:1232–1245.
43. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*. 1997;17:2479–2488.
44. Tian J, Yan Z, Wu Y, Zhang SL, Wang K, Ma XR, Guo L, Wang J, Zuo L, Liu JY, Quan L, Liu HR. Inhibition of iNOS protects endothelial-dependent vasodilation in aged rats. *Acta Pharmacol Sin*. 2010;31:1324–1328.
45. Gunnett CA, Lund DD, Chu Y, Brooks RM 2nd, Faraci FM, Heistad DD. NO-dependent vasorelaxation is impaired after gene transfer of inducible NO-synthase. *Arterioscler Thromb Vasc Biol*. 2001;21:1281–1287.
46. Boura-Halfon S, Zick Y. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab*. 2009;296:E581–E591.
47. Fitzgibbons TP, Kogan S, Aouadi M, Hendricks GM, Straubhaar J, Czech MP. Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation. *Am J Physiol Heart Circ Physiol*. 2011;301:H1425–H1437.
48. Police SB, Thatcher SE, Charnigo R, Daugherty A, Cassis LA. Obesity promotes inflammation in periaortic adipose tissue and angiotensin II-induced abdominal aortic aneurysm formation. *Arterioscler Thromb Vasc Biol*. 2009;29:1458–1464.
49. Marchesi C, Ebrahimian T, Angulo O, Paradis P, Schiffrin EL. Endothelial nitric oxide synthase uncoupling and perivascular adipose oxidative stress and inflammation contribute to vascular dysfunction in a rodent model of metabolic syndrome. *Hypertension*. 2009;54:1384–1392.
50. Meijer RI, Bakker W, Alta CL, Sipkema P, Yudkin JS, Viollet B, Richter EA, Smulders YM, van Hinsbergh VW, Serné EH, Eringa EC. Perivascular adipose tissue control of insulin-induced vasoreactivity in muscle is impaired in db/db mice. *Diabetes*. 2013;62:590–598.

Significance

Perivascular adipose tissue surrounds most arteries and has anticontractile function. Increased inflammation in the vasculature-associated perivascular adipose tissue is present during hypertension, atherosclerosis, and obesity. Our study reveals a yet unexplored function of rictor, an essential component of mammalian target of rapamycin complex 2, in perivascular adipocytes to regulate anticontractility via inhibiting the expression of inflammatory cytokines and inducible nitric oxide synthase. Our results suggest a direct gate keeper function for perivascular adipose mammalian target of rapamycin complex 2 in mediating vascular inflammation. The present findings underscore the importance to address mammalian target of rapamycin complex 2 signaling during inflammation-induced vascular damage.

PNAS

Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21

Marion Cornu[a], Wolfgang Oppliger[a], Verena Albert[a], Aaron M. Robitaille[a], Francesca Trapani[b], Luca Quagliata[b], Tobias Fuhrer[c], Uwe Sauer[c], Luigi Terracciano[b], and Michael N. Hall[a,1]

[a]Biozentrum, University of Basel, CH-4056 Basel, Switzerland; [b]Institute of Pathology, Molecular Pathology Division, University Hospital of Basel, CH-4003 Basel, Switzerland; and [c]Institute of Molecular Systems Biology, Swiss Federal Institute of Technology (ETH) Zürich, CH-8093 Zurich, Switzerland

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2014.

Contributed by Michael N. Hall, June 27, 2014 (sent for review May 20, 2014)

The liver is a key metabolic organ that controls whole-body physiology in response to nutrient availability. Mammalian target of rapamycin (mTOR) is a nutrient-activated kinase and central controller of growth and metabolism that is negatively regulated by the tumor suppressor tuberous sclerosis complex 1 (TSC1). To investigate the role of hepatic mTOR complex 1 (mTORC1) in whole-body physiology, we generated liver-specific *Tsc1* (L-*Tsc1* KO) knockout mice. L-*Tsc1* KO mice displayed reduced locomotor activity, body temperature, and hepatic triglyceride content in a rapamycin-sensitive manner. Ectopic activation of mTORC1 also caused depletion of hepatic and plasma glutamine, leading to peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α)–dependent fibroblast growth factor 21 (FGF21) expression in the liver. Injection of glutamine or knockdown of PGC-1α or FGF21 in the liver suppressed the behavioral and metabolic defects due to mTORC1 activation. Thus, mTORC1 in the liver controls whole-body physiology through PGC-1α and FGF21. Finally, mTORC1 signaling correlated with FGF21 expression in human liver tumors, suggesting that treatment of glutamine-addicted cancers with mTOR inhibitors might have beneficial effects at both the tumor and whole-body level.

TSC | hepatocellular carcinoma | metabolic stress | behavior

The atypical Ser/Thr kinase target of rapamycin (TOR) is a central controller of cell growth and metabolism, conserved from yeast to human. TOR exists in two structurally and functionally distinct complexes, TORC1 and TORC2 (1-4). Mammalian TOR complex 1 (mTORC1) consists of mTOR, raptor, and mLST8. mTORC1 is activated by nutrients, growth factors, and cellular energy and is acutely inhibited by rapamycin. Growth factors activate mTORC1 via the PI3K-PDK1-Akt signaling pathway. Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC) heterodimer TSC1-TSC2. The TSC complex is a GTPase activating protein (GAP) toward the small GTPase ras-homolog enriched in brain (Rheb) that directly binds and activates mTORC1. Thus, deletion of either *Tsc1* or *Tsc2* causes ectopic activation of mTORC1. mTORC1 promotes anabolic processes such as protein, lipid, and nucleotide synthesis and ribosome biogenesis and inhibits catabolic processes such as autophagy (4–8). The best-characterized substrates of mTORC1 are 4E-BP and S6 kinase (S6K). Deregulation of the mTOR signaling network is associated with aging and several diseases, including diabetes, obesity, and cancer (9–11). In the tumor syndromes tuberous sclerosis complex and lymphangioleiomyomatosis (LAM), mTORC1 is deregulated due to mutations in the tumor suppressor gene *Tsc1* or *-2*. Rapamycin and rapamycin analogs (rapalogs) are currently used as immunosuppressive agents and as anticancer drugs (12, 13).

In mammals, behavior and physiology display 24-h oscillations controlled by environmental cues such as light and feeding. Light activates the suprachiasmatic nucleus (SCN) in the hypothalamus. The SCN synchronizes cells in other brain regions and peripheral organs, such as the liver, either by humoral and neuronal signals (14) or by regulating body temperature and the fasting/feeding cycle (15–18). The fasting/feeding cycle is a particularly important synchronizer (*Zeitgeber*) of the liver (19, 20). Expression of several hepatic enzymes and hormones involved in carbohydrate, lipid, cholesterol, and xenobiotic metabolism are regulated over a 24-h cycle in response to nutrient availability (21–28). However, the molecular mechanism(s) by which nutrients control behavior and metabolism is poorly understood but may involve TORC1, a nutrient sensor and key regulator of metabolism. In *Drosophila*, neuronal TORC1 signaling affects circadian behavior (29). In the SCN, mTORC1 signaling is activated by light and controls behavior in a circadian manner (30–32). In the liver, fasting/feeding cycles regulate the energy sensor AMPK and mTORC1 (24, 33–36). Hepatic mTORC1 and the NAD^+-dependent deacetylase SIRT1 are active during the night whereas AMPK is active during the day (34, 35, 37, 38). Moreover, in the liver and other tissues, AMPK (35) and SIRT1 (37, 38) control the core clock machinery. The core clock machinery, composed of the transcription factors CLOCK, BMAL1, PER, CRY, ROR, and REV-ERB, determines circadian oscillations. Disruption of core clock components in the liver strongly alters the regulation of hepatic as well as whole-body glucose and lipid metabolism (39–41). Importantly, time of feeding profoundly affects hepatic gene expression as well as mTORC1 and AMPK signaling (24, 33). Thus, time-restricted feeding improves metabolic rhythms and protects against obesity and liver diseases (33). However, the

Significance

The mammalian target of rapamycin complex 1 (mTORC1) controls cell growth and metabolism in response to nutrients, growth factors, and cellular energy. Aberrant mTORC1 signaling is implicated in human diseases such as diabetes, obesity, and cancer. Our results reveal that ectopic mTORC1 activation in the liver controls the stress hormone fibroblast growth factor 21 (FGF21) in a peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α)–dependent manner via glutamine depletion, which in turn affects whole-body behavior and metabolism. mTORC1 signaling correlates with FGF21 expression in human liver tumors, suggesting that our findings in mice may have physiological relevance in glutamine-addicted human cancers. Thus, treatment with the anticancer drug rapamycin may have beneficial effects by blocking tumor growth and by preventing deregulation of whole-body physiology due to FGF21 expression.

Author contributions: M.C., V.A., and M.N.H. designed research; M.C., W.O., V.A., A.M.R., F.T., L.Q., and T.F. performed research; U.S. and L.T. contributed new reagents/analytic tools; M.C., F.T., L.Q., T.F., and M.N.H. analyzed data; and M.C. and M.N.H. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. Email: M.Hall@unibas.ch.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1412047111/-/DCSupplemental.

role of hepatic mTORC1 in the control of whole-body behavior and metabolism has not been investigated.

FGF21 is a hormone produced mainly in the liver (42) and whose expression oscillates over a 24-h cycle (43–45). Upon fasting, peroxisome proliferator-activated receptor α (PPARα) (46–48) and possibly PPARγ coactivator (PGC)-1α (49) activate FGF21 expression in the liver. FGF21 in turn controls behavior and whole-body metabolism by acting on the nervous system and peripheral organs (46, 47, 50–52). Via the central nervous system, FGF21 decreases locomotor activity and body temperature. In the liver, FGF21 stimulates fatty-acid oxidation and gluconeogenesis. Thus, the liver modulates glucose and lipid metabolism, locomotor activity, and body temperature, at least in part, through FGF21.

Glutamine, the most abundant amino acid in the body, plays an important role in growth and metabolism. A TSC1 or TSC2 deficiency causes metabolic/energetic stress by increasing anabolic processes and thereby increasing energy consumption (53, 54). Glucose-limited TSC-deficient cells are addicted to glutamine as an alternative carbon source (54). In addition to being a carbon source used for energy generation, glutamine is also a precursor for nucleotides and other amino acids. Finally, glutaminolysis both promotes mTORC1 signaling (55) and is activated by mTORC1 (56). mTORC1 activates glutaminolysis through repression of the glutamate dehydrogenase inhibitor SIRT4 (56). The above accounts, at least in part, for the observation that cancer cells are often addicted to glutamine (57–59).

Here, we investigate the role of hepatic mTORC1 in the regulation of whole-body physiology. Our results demonstrate that hepatic mTORC1 activation, due to *Tsc1* knockout specifically in the liver, causes glutamine depletion and thereby PGC-1α–dependent FGF21 expression. This in turn leads to decreased locomotor activity, body temperature, and hepatic lipid content. Thus, hepatic mTORC1 controls behavior and lipid metabolism through FGF21. Furthermore, our findings suggest that glutamine-addicted tumors deregulate whole-body behavior and metabolism.

Results

Hepatic mTORC1 Controls Locomotor Activity, Body Temperature, and Lipid Metabolism. To investigate the role of hepatic mTORC1 in whole-body physiology, we generated mice lacking *Tsc1* exclusively in hepatocytes (L-*Tsc1* KO mice). The L-*Tsc1* KO mice displayed reduced levels of TSC1 and TSC2 specifically in the liver (Fig. 1*A* and Fig. S1*A*). The decrease in TSC2 is consistent with previous reports indicating that TSC1 stabilizes TSC2 (60, 61). Knockout mice fed ad libitum exhibited unchanged body weight and composition, compared with controls (Fig. S1 *B* and *C*). Mice were also subjected to fasting and refeeding to evaluate the effect of *Tsc1* knockout on mTORC1 signaling. The L-*Tsc1* KO mice displayed constitutively active mTORC1 signaling in the liver, as indicated by high levels of S6 phosphorylation upon both fasting and feeding. Akt phosphorylation was significantly reduced in refed L-*Tsc1* KO mice, as expected due to both the S6K-mediated negative feedback loop (62–64) and ER stress (65, 66) (Fig. 1*A*). We next examined the effect of constitutive hepatic mTORC1 signaling on glucose and lipid homeostasis. Blood-glucose levels and hepatic expression of the gluconeogenic gene *G6Pase* were increased in fasted L-*Tsc1* KO mice (Fig. S1 *D* and *E*). Triglyceride content was reduced in the liver of L-*Tsc1* KO mice upon both fasting and refeeding (Fig. 1*B*). Thus, constitutive hepatic mTORC1 signaling disrupts glucose and lipid homeostasis. As shown by Yecies et al., the decreased hepatic triglyceride content observed in refed L-*Tsc1* KO mice is due to attenuation of Akt signaling by the negative feedback loop (67) (Fig. 1*A*). To better understand the decrease in hepatic triglyceride content observed in fasted L-*Tsc1* KO mice, we measured expression of *Pgc-1α* involved in mitochondrial oxidation and/or biogenesis. Consistent with the observed decrease in triglyceride content, expression of *Pgc-1α* and the PGC-1α target gene *CD36* was increased twofold specifically in fasted knockout mice (Fig. S1*E*). This suggests that the decrease in triglyceride levels in fasted mice was due to an increase in fatty acid oxidation. The above results are in agreement with previous studies (68, 69), thereby confirming our L-*Tsc1* knockout.

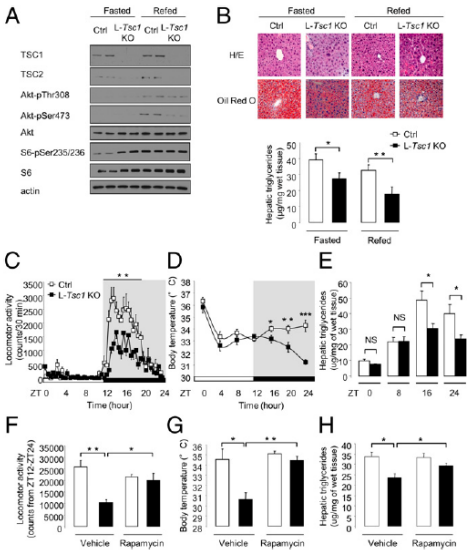


Fig. 1. Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism. (*A*) Immunoblots of liver extracts from L-*Tsc1* KO and control mice fasted overnight or refed for 4 h. Each lane consists of a mixture of liver extracts obtained from three animals. (*B*) Representative images of hematoxylin/eosin (H&E) and Oil red O staining of liver sections from L-*Tsc1* KO and control mice fasted overnight ($n = 8$ for control and $n = 7$ for L-*Tsc1* KO) or refed for 4 h ($n = 9$ for control and $n = 7$ for L-*Tsc1* KO). [Original magnification: 40× (*Upper*).] Hepatic triglyceride content was measured from L-*Tsc1* KO and control mice fasted overnight ($n = 8$ for control and $n = 7$ for L-*Tsc1* KO) or refed for 4 h (control $n = 9$ and L-*Tsc1* KO $n = 7$) (*Lower*). (*C–H*) Food was removed at ZT0. ZT is *Zeitgeber* time within a 24-h light/dark cycle, with ZT0 and ZT12 corresponding to the appearance and disappearance of light, respectively. (*C* and *D*) Locomotor activity was measured by the Comprehensive Laboratory Animal Monitoring System (CLAMS) (*C*), and body temperature was measured by rectal thermometer every 4 h over a 24-h cycle (*D*) ($n = 8$ per group). (*E*) Hepatic triglyceride content from L-*Tsc1* KO and control mice was measured ($n = 6$ per time point and per genotype). (*F*) Locomotor activity was measured during the dark phase in L-*Tsc1* KO and control mice. Animals were treated with rapamycin (2 mg/kg) or vehicle at ZT11.5. Data are presented as total counts from ZT12 to ZT24 ($n = 6$ per group). (*G*) Body temperature was measured by rectal thermometer at ZT24 in L-*Tsc1* KO and control mice. Animals were treated with rapamycin (2 mg/kg) or vehicle at ZT18 ($n = 6$ per group). (*H*) Hepatic triglyceride content from L-*Tsc1* KO and control mice at ZT20. Animals were treated with rapamycin (2 mg/kg) or control vehicle at ZT14 ($n = 6$ per group). White bars or squares represent control mice and black bars or squares represent L-*Tsc1* KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

The above results taken together indicate that loss of TSC confers a phenotype mainly under fasted conditions. This is expected because the TSC complex inhibits mTORC1 under fasted, but not fed, conditions. Because loss of TSC confers a phenotype mainly under starvation conditions, all subsequent

experiments designed to investigate the effect of mTORC1 activation on whole-body physiology were performed in fasted mice. Furthermore, examining only fasted mice circumvents complications due to the negative feedback loop that inhibits Akt during feeding.

To examine the role of hepatic mTORC1 in behavior and lipid metabolism, L-*Tsc1* KO and control mice were monitored for locomotor activity, body temperature, and hepatic triglyceride content over a 24-h cycle. The mice were fasted for the entire 24 h starting at ZT0. ZT is *Zeitgeber* time within a 24-h light/dark cycle, with ZT0 and ZT12 corresponding to the appearance and disappearance of light, respectively. We note that, in our experimental conditions, ZT24 corresponds to 24 h of fasting and is thus different from ZT0. L-*Tsc1* KO mice displayed reduced locomotor activity and body temperature during the dark phase, compared with controls (Fig. 1 *C* and *D*). Body temperature decreased by ZT4 in both L-*Tsc1* KO and control mice but dropped further during the dark phase in knockout mice (Fig. 1*D*). Plasma levels of thyroid (T4) hormone and adipose-secreted leptin, key regulators of locomotor activity and thermogenesis, were similar in L-*Tsc1* KO and control mice (Fig. S1*F*). Thus, mTORC1 appears to control locomotor activity and body temperature independently of leptin and thyroid hormone. Similar to locomotor activity and body temperature, triglyceride levels were reduced during the dark phase in the knockout mice (Fig. 1*E*), suggesting that mTORC1 also controls the daily levels of hepatic lipid metabolism.

To determine whether the observed decrease in locomotor activity, body temperature, and triglycerides was indeed due to mTORC1, animals were treated with rapamycin. The i.p. injection of rapamycin abolished hepatic mTORC1 signaling in L-*Tsc1* KO mice (Fig. S1*G*) and restored normal levels of locomotor activity, body temperature, and hepatic triglycerides (Fig. 1 *F*–*H*). Collectively, these data demonstrate that hepatic mTORC1 controls the daily levels of locomotor activity, body temperature, and lipid metabolism.

To investigate whether mTORC1 signaling itself oscillates, we examined S6 phosphorylation in the liver of wild-type mice killed every 4 h over a 24-h cycle. S6 phosphorylation was low during the light phase and high during the dark phase, indicating that mTORC1 signaling oscillates daily, even in the absence of food (Fig. 2*A*). This is in agreement with previous findings (34). To determine the role of the TSC complex in the daily regulation of mTORC1 signaling, we next examined hepatic S6 phosphorylation in L-*Tsc1* KO mice. S6 phosphorylation displayed the similar diurnal rhythm, but at higher absolute levels at all time points, compared with the wild-type control (Fig. 2*A* and Fig. S1*H*). Thus, mTORC1 signaling oscillates daily, and this regulation is independent of the TSC complex.

Hepatic mTORC1 Controls FGF21 Expression. How does hepatic mTORC1 control locomotor activity, body temperature, and lipid metabolism? Several observations suggest that mTORC1 may control behavior and lipid metabolism via FGF21. The hormone FGF21, expressed mainly in the liver, decreases locomotor activity, body temperature, and hepatic lipid accumulation (46, 47, 51). Furthermore, mTORC1 activates PGC-1α (Fig. S1*E*), and PGC-1α has been shown to promote FGF21 expression (49). To determine whether mTORC1 controls FGF21 expression, we examined *Fgf21* mRNA levels in the liver and FGF21 protein levels in plasma in L-*Tsc1* KO and control mice killed every 4 h over a 24-h cycle. Similar to mTORC1 activity (Fig. 2*A*), FGF21 expression was low during the light phase and high during the dark phase in both L-*Tsc1* KO and control mice, but the increase during the dark phase was significantly higher in the knockout mice (Fig. 2 *B* and *C*). The i.p. injection of rapamycin restored normal *Fgf21* mRNA levels in L-*Tsc1* KO mice (Fig. 2*D*). The above results

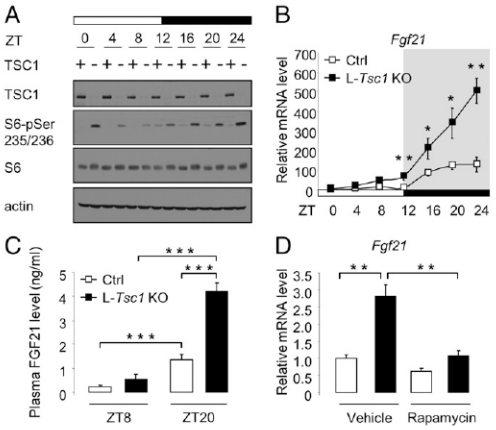


Fig. 2. Hepatic mTORC1 controls FGF21 expression. (*A* and *B*) Mice were fasted at ZT0 and killed every 4 h over a 24-h cycle. (*A*) Immunoblots of total liver extracts from L-*Tsc1* KO and control mice. Each lane consists of a mixture of total liver extracts obtained from six animals. (*B*) Expression of *Fgf21* mRNA in the liver of L-*Tsc1* KO and control mice was measured by quantitative reverse transcription PCR (qRT-PCR). Total liver RNA was extracted. White squares represent control mice, and black squares represent L-*Tsc1* KO mice ($n = 6$ per time point and per genotype). (*C*) Plasma FGF21 levels in L-*Tsc1* KO and control mice. Food was removed at ZT0, and plasma FGF21 levels were measured at ZT8 and ZT20. White bars represent control mice, and black bars represent L-*Tsc1* KO mice ($n = 6$ per time point and per genotype). (*D*) Expression of *Fgf21* mRNA at ZT20 in the liver of L-*Tsc1* KO and control mice was measured by qRT-PCR. Food was removed at ZT0. Animals were treated with rapamycin (2 mg/kg) or vehicle at ZT14. Total liver RNA was prepared from L-*Tsc1* KO and control animals. White bars represent control mice, and black bars represent L-*Tsc1* KO mice ($n = 6$ per condition and per genotype). Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

suggest that hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism via FGF21 expression.

Core clock components, in addition to FGF21, are also key regulators of locomotor activity, body temperature, and hepatic glucose and lipid metabolism (40, 41). In agreement with previous studies, *Cry1*, *Cry2*, *Per1*, *Per2*, *Rorα*, and *Rorγ* mRNA levels and CRY2 and PER2 protein levels in the liver were low during the light phase and high during the dark phase in control animals (Fig. S2 *A* and *B*) (70–72). Also in agreement with previous studies, we observed that *Rev-erbα* and *Rev-erbβ* expression was the inverse of the above (Fig. S2 *A* and *B*) (70, 71, 73). L-*Tsc1* KO mice displayed little-to-no change in (*i*) expression of the core clock components, as measured at the mRNA and protein levels (Fig. S2 *A* and *B*), (*ii*) interaction of circadian clock components (Fig. S2*C*), and (*iii*) binding of CLOCK to the *fgf21* promoter (Fig. S3). Thus, mTORC1 appears not to control the core clock components, again suggesting that mTORC1 may control whole-body physiology through FGF21.

Hepatic mTORC1 Controls Locomotor Activity, Body Temperature, and Lipid Metabolism Through FGF21. To investigate whether FGF21 is responsible for the decreased locomotor activity, body temperature, and triglycerides in L-*Tsc1* KO mice, we examined the effect of FGF21 knockdown. L-*Tsc1* KO and control mice were infected with adenovirus expressing shRNA against FGF21. The knockdown was confirmed by loss of FGF21 mRNA in the liver and reduced FGF21 protein in plasma (Fig. 3 *A* and *B*). Knockdown of

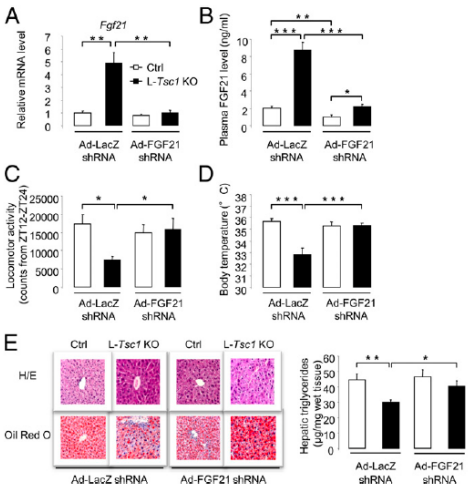


Fig. 3. Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21. (*A–E*) L-*Tsc1* KO and control mice were infected with an adenovirus expressing shRNA against FGF21 or LacZ. Four days after infection, food was removed at ZT0, and animals were killed at ZT24 for further measurements. (*A* and *B*) *Fgf21* mRNA (*A*) and plasma levels (*B*) were measured ($n = 6$ per condition and per genotype). (*C*) Locomotor activity was measured during the dark phase. Data are presented as total counts from ZT12 to ZT24 ($n = 6$ per condition and per genotype). (*D*) Body temperature was measured by rectal thermometer ($n = 6$ per condition and per genotype). (*E*) Representative images of hematoxylin/eosin (H&E) and Oil red O staining of liver sections. [Original magnification: 40× (*Left*).] Hepatic triglyceride content was measured (*Right*) ($n = 6$ per condition and per genotype). White bars represent control mice, and black bars represent L-*Tsc1* KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

FGF21 restored normal locomotor activity, body temperature, and hepatic triglycerides in L-*Tsc1* KO mice (Fig. 3 *C–E*). Thus, hepatic mTORC1 inhibits locomotor activity, body temperature, and lipid metabolism through FGF21.

FGF21 controls carbohydrate metabolism in the liver (50) by inducing expression of gluconeogenic genes (74–76). L-*Tsc1* KO mice display an increase in expression of gluconeogenic genes in the liver and increased blood glucose levels (Fig. S1 *D* and *E*). We investigated whether mTORC1 controls glucose metabolism through FGF21. Knockdown of FGF21 in the liver had no effect on blood glucose levels or expression of *Pgc-1α*, *G6Pase*, and *PEPCK* (Fig. S4 *A* and *B*). Thus, mTORC1 controls carbohydrate metabolism independently of FGF21.

Hepatic mTORC1 Controls FGF21 and Behavior and Lipid Metabolism Through PGC-1α. How does hepatic mTORC1 control FGF21 expression? Purushotham et al. (49) reported that the transcriptional coactivator PGC-1α promotes FGF21 expression, and our results (Fig. S1*E*) indicate that *Pgc-1α* is up-regulated in L-*Tsc1* KO mice. Furthermore, PGC-1α controls locomotor activity and body temperature (77) and promotes expression of genes involved in mitochondrial fatty acid oxidation, thereby preventing hepatic steatosis (78–80). Thus, we investigated whether hepatic mTORC1 controls FGF21 and whole-body physiology via PGC-1α. First, we examined PGC-1α mRNA and protein levels in the liver of L-*Tsc1* KO and control mice killed every 4 h over a 24-h cycle. PGC-1α expression was low during the light phase and high during the dark phase, reaching a peak at ZT20 in both L-*Tsc1* KO and control mice, but was significantly increased overall in the knockout compared with the control (Fig. 4 *A* and *B*). Expression of PGC-1α target genes, such as *Fgf21* and the gluconeogenic genes *G6Pase* and *PEPCK*, was also low during the light phase and high during the dark phase (Figs. 2*B* and 4*C*). Second, we determined whether i.p. injection of rapamycin reduced expression of *Pgc-1α* and *G6Pase* in the liver of L-*Tsc1* KO mice (Fig. 4*D*). As observed

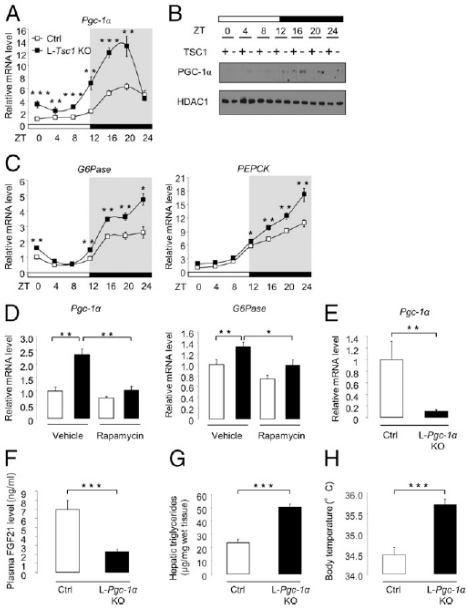


Fig. 4. Hepatic PGC-1α controls FGF21 expression. (*A–C*) Mice were fasted at ZT0 and killed every 4 h over a 24-h cycle. (*A*) Expression of *Pgc-1α* mRNA in the liver of L-*Tsc1* KO and control mice was measured by qRT-PCR. Total liver RNA was extracted. White squares represent control mice, and black squares represent L-*Tsc1* KO mice ($n = 6$ per time point and per genotype). (*B*) Immunoblots of liver nuclear extracts from L-*Tsc1* KO and control mice. PGC-1α protein levels were analyzed. Each lane consists of a mixture of liver nuclear extracts obtained from six animals per genotype. (*C*) Expression of *G6Pase* and *PEPCK* mRNA in the liver of L-*Tsc1* KO and control mice was measured by qRT-PCR. Total liver RNA was extracted. White squares represent control mice, and black squares represent L-*Tsc1* KO mice ($n = 6$ per time point and per genotype). (*D*) Expression of *Pgc-1α*, *G6Pase* mRNA at ZT20 in the liver of L-*Tsc1* KO and control mice was measured by qRT-PCR. Food was removed at ZT0, and animals were treated with rapamycin (2 mg/kg) or vehicle at ZT14. Total liver RNA was extracted. White bars represent control mice, and black bars represent L-*Tsc1* KO mice ($n = 6$ per condition and per genotype). (*E–H*) *Pgc-1α* floxed mice were infected with a Cre (L-*Pgc-1α* KO) or null (Ctrl)-expressing adenovirus. Four days after infection, animals were fasted at ZT0 for 20 h before being killed. White bars represent control mice ($n = 7$), and black bars represent L-*Pgc-1α* KO mice ($n = 8$). (*E*) Expression of *Pgc-1α* mRNA in the liver was measured by qRT-PCR. (*F*) Plasma FGF21 levels were measured. (*G*) Hepatic triglyceride content was measured. (*H*) Body temperature was measured by rectal thermometer at ZT20. Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

previously for *Fgf21* expression (Fig. 2*D*), rapamycin restored normal expression of *Pgc-1α* and *G6Pase*. Third, we examined FGF21 expression upon knockout of *Pgc-1α* in the liver. Mice containing a floxed *Pgc-1α* allele were infected with Cre-expressing adenovirus. Hepatic deletion of *Pgc-1α* (L-*Pgc-1α* KO) was confirmed by loss of *Pgc-1α* mRNA in the liver (Fig. 4*E*). L-*Pgc-1α* KO mice displayed a strong decrease in FGF21 protein in the plasma (Fig. 4*F*), a twofold increase in triglyceride content in the liver (Fig. 4*G*), and a >1 °C increase in body temperature (Fig. 4*H*). Fourth, we examined the effect of PGC-1α knockdown in L-*Tsc1* KO mice. Infection of L-*Tsc1* KO mice with adenovirus expressing shRNA against PGC-1α strongly decreased hepatic *Pgc-1α* expression and reduced expression of *G6Pase*, *PEPCK*, and *CD36* (Fig. 5*A* and Fig. S5). Importantly, knock down of *Pgc-1α* also decreased FGF21 expression, as measured at both the mRNA and plasma protein level (Fig. 5 *A* and *B*), and restored normal locomotor activity, body temperature, and hepatic triglyceride content in L-*Tsc1* KO mice (Fig. 5 *C*–*E*). Collectively, the above results suggest that mTORC1 controls FGF21 and ultimately behavior and lipid metabolism via PGC-1α.

The transcriptional repressor E4BP4 inhibits FGF21 expression by binding a D-box in the *Fgf21* promoter (45). Although *E4BP4* expression increased in the dark phase, its expression was similar in L-*Tsc1* KO and control mice (Fig. S6*A*). ER stress promotes FGF21 expression through ATF4 expression (81–83). ATF4 is a transcriptional effector of the PERK-branch of the unfolded protein response. L-*Tsc1* KO mice displayed an early ER stress response as assessed by eIF2α phosphorylation (Fig. S6*B*) (84). However, ATF4 expression was not affected in L-*Tsc1* KO mice, as measured at both the mRNA and protein level (Fig. S6*C*). Thus, mTORC1 does not appear to regulate FGF21 via E4BP4 or ATF4, further suggesting that mTORC1 activates FGF21 through PGC-1α. Of note, L-*Tsc1* KO mice displayed similar *PPARα* mRNA levels compared with controls, suggesting that PPARα is not the limiting factor controlling FGF21 expression (Fig. S6*D*).

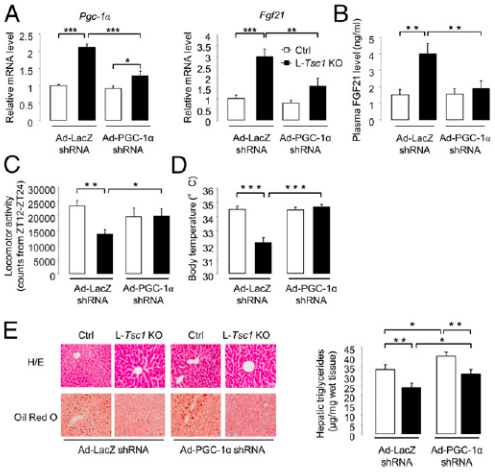


Fig. 5. Hepatic mTORC1 controls FGF21 and behavior and lipid metabolism through Pgc-1α. (*A*–*E*) L-*Tsc1* KO and control mice were infected either with an adenovirus expressing shRNA against PGC-1α or LacZ. Four days after infection, food was removed at ZT0. (*A* and *B*) *Pgc-1α* and *Fgf21* mRNA (*A*) and plasma FGF-21 levels (*B*) were measured at ZT20 ($n = 10$ per condition and per genotype). (*C*) Locomotor activity was measured during the dark phase. Data are presented as total counts between ZT12 and ZT24 ($n = 8$ per condition and per genotype). (*D*) Body temperature was measured by rectal thermometer at ZT24 ($n = 8$ per condition and per genotype). (*E*) Representative images of hematoxylin/eosin (H&E) and Oil red O staining of liver sections. Animals were killed at ZT24. [Original magnification: 40× (*Left*).] Hepatic triglyceride content was measured (*Right*) ($n = 8$ per condition and per genotype). White bars represent control mice, and black bars represent L-*Tsc1* KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Hepatic mTORC1 Activates PGC-1α and FGF21 via Glutamine Depletion. The hormone FGF21 is produced in the liver (42) in response to metabolic stress such as carbon depletion (46, 47, 83, 85). Glutamine, via glutaminolysis, can serve as an alternative carbon source to prevent metabolic/energetic stress in glucose-deprived cells (53, 54, 59). mTORC1 promotes glutaminolysis, and consequently mTORC1 hyperactivation in *Tsc2*-deficient cells leads to glutamine depletion (56). Thus, we investigated whether L-*Tsc1* KO mice display glutamine depletion that may in turn induce FGF21. First, we examined glutamine levels in the liver and in plasma of L-*Tsc1* KO mice. Glutamine levels were decreased during the dark phase in both L-*Tsc1* KO and control mice, but the decrease in the dark phase was significantly more pronounced in the knockout mice (Fig. 6*A*). Asparagine is synthesized by amide transfer from glutamine to aspartate. Consistent with the reduced hepatic glutamine levels, asparagine levels were also reduced in the liver whereas aspartate levels were unaffected (Fig. S7*A*). The levels of branched-chain amino acids were also unchanged (Fig. S7*A*). Thus, L-*Tsc1* KO mice are depleted specifically for glutamine. Second, to determine whether glutamine depletion was due to mTORC1 hyperactivity, animals were treated with rapamycin. The i.p. injection of rapamycin restored normal glutamine levels in L-*Tsc1* KO mice (Fig. 6 *B* and *C*). Third, we examined whether glutamine depletion is responsible for PGC-1α and FGF21 expression. The i.p. injection of glutamine decreased *Pgc-1α* and *Fgf21* mRNA levels in the liver, reduced FGF21 protein levels in plasma, and increased body temperature (Fig. 6 *D*–*F*). Interestingly, glutamine also increased hepatic mTORC1 signaling in both L-*Tsc1* KO and control mice (Fig. 6*G*), consistent with previous in vitro studies (55, 86). Collectively, these data suggest that hepatic mTORC1 controls whole-body glutamine levels and thereby whole-body physiology through PGC-1α–dependent FGF21 expression.

mTORC1 Signaling Correlates with FGF21 Expression in Human Liver Tumors. Several observations suggest that mTORC1, often deregulated in cancer, may lead to FGF21 expression in tumors. First, as described in Fig. 6 *A*–*C* and in the literature (56), mTORC1 hyperactivation causes glutamine depletion. Second, tumors are often glutamine-addicted, causing cancer patients to display glutamine depletion (57–59). Third, FGF21 is expressed in response to metabolic stress such as carbon limitation (46, 47, 83, 85). To determine whether mTORC1 signaling correlates with FGF21 expression in tumors, we examined S6 phosphorylation and FGF21 protein levels in human hepatocellular carcinoma (HCC) biopsies. Histological examination of HCC from 10 separate patients revealed a strong correlation between mTORC1 signaling and FGF21 expression. Four HCCs expressed both phospho-S6 and FGF21 whereas six expressed neither (Fig. 6*H* and Fig. S7*B*). Thus, our findings in L-*Tsc1* KO mice may be physiologically relevant in tumors and, furthermore, suggest that treatment of glutamine-addicted cancers with mTOR inhibitors might have beneficial effects at both the tumor and whole-body level.

Discussion

To obtain insight on how nutrient availability controls whole-body behavior and metabolism, we investigated the role of the nutrient sensor mTORC1 on locomotor activity, body temperature, and glucose and lipid homeostasis. In particular, we examined the

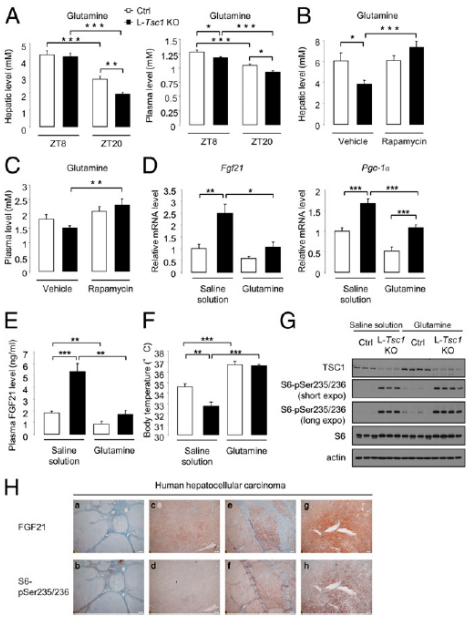


Fig. 6. Hepatic mTORC1 activates PGC-1α and FGF21 via glutamine depletion. (*A*) Hepatic and plasma glutamine levels in L-*Tsc1* KO and control mice were quantified by LC-MS/MS. 8–9-wk-old mice were fasted at ZT0 and killed at ZT8 and ZT20 ($n = 6$ per time point and per genotype). (*B*) Hepatic glutamine levels in L-*Tsc1* KO and control mice were quantified by LC-MS/MS. 12-wk-old animals were fasted at ZT0, treated with rapamycin (2 mg/kg) or control vehicle and killed at ZT20 ($n = 6$ per condition and per genotype). (*C*) Plasma glutamine levels in L-*Tsc1* KO and control mice were quantified by LC-MS/MS. Twelve-week-old animals were fasted at ZT0, treated with rapamycin (2 mg/kg) or control vehicle and killed at ZT20 ($n = 6$ per condition and per genotype). (*D–G*) Food was removed at ZT0 and L-*Tsc1* KO mice and controls were treated with L-Glutamine (1 g/kg) or saline solution, 2 h before sacrifice at ZT20. (*D*) Expression of *Fgf21* and *Pgc-1α* mRNA in the liver of L-*Tsc1* KO and control mice was measured by qRT-PCR. Total liver RNA was extracted ($n = 6$ per condition and per genotype). (*E*) Plasma FGF21 levels in L-*Tsc1* KO and control mice were measured ($n = 6$ per condition and per genotype). (*F*) Body temperature was measured by rectal thermometer at ZT20 ($n = 6$ per condition and per genotype). (*G*) Immunoblots of liver extracts from L-*Tsc1* KO and control mice injected with glutamine or saline solution. (*H*) FGF21 and phospho-S6 staining in human HCC. Representative images of an HCC showing little-to-no staining of either (*a*) FGF21 or (*b*) phospho-S6. Images of HCC presenting diffuse staining of both (*c*) FGF21 and (*d*) phospho-S6. (*e–h*) Examples of HCCs exhibiting medium to high staining of both (e and *g*) FGF21 and (*f* and *h*) phospho-S6. White bars represent control mice, and black bars represent L-*Tsc1* KO mice. Values are expressed as mean ± SEM; the * indicates statistical significant difference between the indicated groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

effects of hepatic mTORC1 activation and inhibition on whole-body physiology using L-*Tsc1* KO or rapamycin-treated mice, respectively. We observed that mTORC1 hyperactivity in the liver induced glutamine depletion, which in turn activated the transcriptional coactivator PGC-1α and thereby the stress hormone FGF21. FGF21 expression reduced locomotor activity, body temperature, and lipid metabolism. Our results suggest that mTORC1 in the liver controls hepatic metabolism and whole-body behavior (Fig. 7). Finally, we found that mTORC1 signaling in human HCC correlates with FGF21 expression. This suggests that our findings in mice may have physiological relevance in glutamine-addicted human cancers. Glutamine-addicted tumors could deregulate whole-body physiology through FGF21. Accordingly, treatment with the anticancer drug rapamycin may have beneficial effects at both the tumor and whole-body level. In addition to blocking growth of the tumor, rapamycin may prevent glutamine depletion and thereby FGF21 expression.

An important observation in this study is that L-*Tsc1* KO mice display whole-body glutamine depletion, and this in turn leads to behavioral and metabolic alterations. The glutamine depletion observed in L-*Tsc1* KO mice and its prevention by rapamycin treatment is consistent with a recent study showing that mTORC1 stimulates glutamine-dependent anaplerosis and that mTORC1 inhibition increases intracellular glutamine levels in cultured cells (56). Moreover, confirming previous in vitro studies (55, 86, 87), we show that glutamine increases hepatic mTORC1 signaling. Thus, our findings indicate that the role of mTORC1 in glutamine metabolism has consequences at the whole-body level in addition to the cellular level. mTORC1 promotes glutamine catabolism that decreases locomotor activity, body temperature, and lipid metabolism. mTOR inhibitors or a glutamine supplement may have a beneficial effect in countering disruption of whole-body physiology due, for example, to disease.

We report that L-*Tsc1* KO mice display an increase in PGC-1α expression. This is consistent with previous studies in skeletal muscle showing a reduction in PGC-1α expression upon inactivation of mTORC1 (88, 89). Although mTORC1 may promote expression of the transcriptional coactivator PGC-1α in both muscle and liver, the molecular mechanism appears to be different in the two tissues. In muscle cells, mTORC1 controls PGC-1α expression through the transcription factor YY1 and under conditions where glutamine is likely not limiting (89). In liver, mTORC1 activates PGC-1α expression in response to glutamine depletion. The mechanism through which glutamine depletion activates *Pgc-1α* remains unknown but might be independent of YY1.

How does PGC-1α activate *Fgf21* in L-*Tsc1* KO mice? PGC-1α is a transcriptional coactivator with RORα/γ or PPARα (77, 79) which have been proposed to be direct activators of *Fgf21*

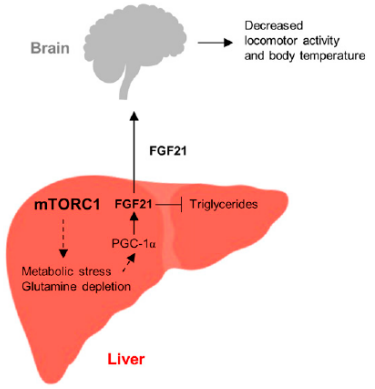


Fig. 7. Model of hepatic mTORC1 controlling whole-body physiology. Hepatic mTORC1 causes glutamine depletion and thereby PGC-1α-dependent FGF21 expression, which in turn affects whole-body physiology.

(46, 90, 91). We found that *RORγ* expression is slightly increased in the liver of L-*Tsc1* KO mice during the dark phase whereas *RORα* and *PPARα* were reduced and unchanged, respectively (Figs. S2*A* and S6*D*). This suggests that PGC-1α may activate *Fgf21* via RORγ but does not rule out PPAR-α.

Our finding that PGC-1α promotes FGF21 expression is consistent with the previous observation that PGC-1α promotes FGF21 through PPARα (49). However, Estall et al., showed that PGC-1α indirectly represses FGF21 through the transcriptional repressor Rev-erbα (90). These seemingly conflicting findings on the role of PGC-1α in the regulation of *Fgf21* may be due to different experimental conditions. We (Fig. S2 *A* and *B*) and others (70, 71, 73) observed that Rev-erbα is expressed only during the light phase whereas glutamine depletion increases PGC-1α and FGF21 expression only during the dark phase. PGC-1α may inhibit FGF21 expression via Rev-erbα during the light phase.

We and others observed that hepatic mTORC1 activity is circadian (24, 33, 34, 36). Two previous studies showed that hepatic mTORC1 rhythmicity is controlled by the circadian clock (34, 36). Furthermore, Jouffe et al. (34) speculated that the circadian clock controls mTORC1 signaling via autophagy. Interestingly, hepatic mTORC1 signaling remains rhythmic in L-*Tsc1* KO mice. It is well established that loss of the TSC complex prevents autophagy, as a consequence of mTORC1 activation, and autophagy is indeed inhibited in L-*Tsc1* KO mice (92). Thus, at least in the absence of the TSC complex, mTORC1 rhythmicity appears not to be regulated by autophagy. Recently, Khapre et al. showed that Bmal1 negatively regulates mTORC1 signaling over a 24-h cycle by affecting the expression of *mtor* and *deptor* in the liver (36). This provides a mechanism through which the transcription factors of the circadian clock impinge on mTORC1 signaling. However, further investigation is required to determine the role of other circadian clock components in the regulation of mTORC1 signaling. The rhythmicity of mTORC1 signaling accounts for the rhythmicity of glutamine depletion and PGC-1α–dependent FGF21 expression. Thus, disruption of the circadian clock frequently observed in cancer or upon jet lag might alter mTORC1 signaling and thereby whole-body physiology. Finally, we note that, although the circadian clock affects mTORC1 signaling (34, 36), mTORC1 itself appears not to control the core clock components (Fig. S2 *A* and *B*).

In conclusion, our findings underscore the importance of hepatic mTORC1 in the regulation of whole-body physiology. Hyperactivation of mTORC1 in the liver causes glutamine depletion, which leads to FGF21 expression and, in turn, changes in whole-body physiology. Moreover, mTORC1 signaling correlates with FGF21 expression in human liver tumors. This latter observation is relevant because human cancers often exhibit hyperactive mTORC1 signaling and glutamine addiction (9–11, 59). Thus, treatment of glutamine-addicted cancers with mTOR inhibitors might have beneficial effects at both the tumor and whole-body level.

Materials and Methods

Mice. Generation of liver-specific Tsc1 knockout (L-*Tsc1* KO) mice has been described previously (6). Where indicated, mice were intraperitoneally injected with rapamycin (LC Laboratories) at 2 mg/kg or vehicle. Rapamycin solvent was composed of 5% (vol/vol) PEG-400, 4% (vol/vol) ethanol, and 5% (vol/vol) Tween 80. Additionally, where indicated, animals were intraperitoneally injected with L-glutamine (Sigma) at 1 g/kg or with saline solution. For details see *SI Materials and Methods*.

Other Methods. For other methods, see *SI Materials and Methods*. The primer sequences used in this study are listed in Table S1.

Statistical Analyses. Statistical significance was measured using a Student's unpaired *t* test to determine differences among two groups. The differences were considered to be significant if $P < 0.05$. Data are presented as mean ± SEM. Immunoblot quantitation was assessed using Image J software.

ACKNOWLEDGMENTS. We thank Christoph Handschin for reagents and Annette Roulier for the illustration. This work was supported by the Swiss National Science Foundation, the Canton of Basel, the Louis Jeantet Foundation (M.N.H.), the Société Francophone du Diabète-Association de Langue Française pour l'Etude du Diabète et des Maladies Métaboliques (M.C.), and the Werner Siemens Foundation (A.M.R. and V.A.).

1. Loewith R, et al. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* 10(3):457–468.
2. Jacinto E, et al. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* 6(11):1122–1128.
3. Sarbassov DD, et al. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 14(14):1296–1302.
4. Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* 124(3):471–484.
5. Laplante M, Sabatini DM (2009) mTOR signaling at a glance. *J Cell Sci* 122(Pt 20): 3589–3594.
6. Robitaille AM, et al. (2013) Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. *Science* 339(6125):1320–1323.
7. Ben-Sahra I, Howell JJ, Asara JM, Manning BD (2013) Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science* 339(6125): 1323–1328.
8. Shimobayashi M, Hall MN (2014) Making new contacts: The mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* 15(3):155–162.
9. Dazert E, Hall MN (2011) mTOR signaling in disease. *Curr Opin Cell Biol* 23(6): 744–755.
10. Cornu M, Albert V, Hall MN (2013) mTOR in aging, metabolism, and cancer. *Curr Opin Genet Dev* 23(1):53–62.
11. Laplante M, Sabatini DM (2012) mTOR signaling in growth control and disease. *Cell* 149(2):274–293.
12. Benjamin D, Colombi M, Moroni C, Hall MN (2011) Rapamycin passes the torch: A new generation of mTOR inhibitors. *Nat Rev Drug Discov* 10(11):868–880.
13. Li J, Kim SG, Blenis J (2014) Rapamycin: One drug, many effects. *Cell Metab* 19(3): 373–379.
14. Cailotto C, et al. (2009) Effects of nocturnal light on (clock) gene expression in peripheral organs: A role for the autonomic innervation of the liver. *PLoS ONE* 4(5): e5650.
15. Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. *Annu Rev Physiol* 72:517–549.
16. Gachon F, Nagoshi E, Brown SA, Ripperger J, Schibler U (2004) The mammalian circadian timing system: From gene expression to physiology. *Chromosoma* 113(3): 103–112.
17. Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol* 12(18):1574–1583.
18. Saini C, Morf J, Stratmann M, Gos P, Schibler U (2012) Simulated body temperature rhythms reveal the phase-shifting behavior and plasticity of mammalian circadian oscillators. *Genes Dev* 26(6):567–580.
19. Damiola F, et al. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14(23): 2950–2961.
20. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. *Science* 291(5503):490–493.
21. Gachon F, Olela FF, Schaad O, Descombes P, Schibler U (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab* 4(1):25–36.
22. Gachon F, Firsov D (2011) The role of circadian timing system on drug metabolism and detoxification. *Expert Opin Drug Metab Toxicol* 7(2):147–158.
23. Sun Z, Lazar MA (2013) Dissociating fatty liver and diabetes. *Trends Endocrinol Metab* 24(1):4–12.
24. Vollmers C, et al. (2009) Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proc Natl Acad Sci USA* 106(50):21453–21458.
25. Huang W, Ramsey KM, Marcheva B, Bass J (2011) Circadian rhythms, sleep, and metabolism. *J Clin Invest* 121(6):2133–2141.
26. Schibler U, Naef F (2005) Cellular oscillators: Rhythmic gene expression and metabolism. *Curr Opin Cell Biol* 17(2):223–229.
27. Eckel-Mahan KL, et al. (2012) Coordination of the transcriptome and metabolome by the circadian clock. *Proc Natl Acad Sci USA* 109(14):5541–5546.
28. Adamovich Y, et al. (2014) Circadian clocks and feeding time regulate the oscillations and levels of hepatic triglycerides. *Cell Metab* 19(2):319–330.
29. Zheng X, Sehgal A (2010) AKT and TOR signaling set the pace of the circadian pacemaker. *Curr Biol* 20(13):1203–1208.

30. Cao R, Anderson FE, Jung YJ, Dziema H, Obrietan K (2011) Circadian regulation of mammalian target of rapamycin signaling in the mouse suprachiasmatic nucleus. *Neuroscience* 181:79–88.
31. Cao R, Lee B, Cho HY, Saklayen S, Obrietan K (2008) Photic regulation of the mTOR signaling pathway in the suprachiasmatic circadian clock. *Mol Cell Neurosci* 38(3): 312–324.
32. Cao R, et al. (2013) Translational control of entrainment and synchrony of the suprachiasmatic circadian clock by mTOR/4E-BP1 signaling. *Neuron* 79(4):712–724.
33. Hatori M, et al. (2012) Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab* 15(6):848–860.
34. Jouffe C, et al. (2013) The circadian clock coordinates ribosome biogenesis. *PLoS Biol* 11(1):e1001455.
35. Lamia KA, et al. (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* 326(5951):437–440.
36. Khapre RV, et al. (2014) BMAL1-dependent regulation of the mTOR signaling pathway delays aging. *Aging (Albany, NY Online)* 6(1):48–57.
37. Asher G, et al. (2008) SIRT1 regulates circadian clock gene expression through PER2 deacetylation. *Cell* 134(2):317–328.
38. Nakahata Y, et al. (2008) The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* 134(2):329–340.
39. Asher G, Schibler U (2011) Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab* 13(2):125–137.
40. Feng D, Lazar MA (2012) Clocks, metabolism, and the epigenome. *Mol Cell* 47(2): 158–167.
41. Eckel-Mahan K, Sassone-Corsi P (2013) Metabolism and the circadian clock converge. *Physiol Rev* 93(1):107–135.
42. Nishimura T, Nakatake Y, Konishi M, Itoh N (2000) Identification of a novel FGF, FGF-21, preferentially expressed in the liver. *Biochim Biophys Acta* 1492(1):203–206.
43. Kornmann B, Schaad O, Reinke H, Saini C, Schibler U (2007) Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb Symp Quant Biol* 72:319–330.
44. Oishi K, Uchida D, Ishida N (2008) Circadian expression of FGF21 is induced by PPARalpha activation in the mouse liver. *FEBS Lett* 582(25-26):3639–3642.
45. Tong X, et al. (2010) Transcriptional repressor E4-binding protein 4 (E4BP4) regulates metabolic hormone fibroblast growth factor 21 (FGF21) during circadian cycles and feeding. *J Biol Chem* 285(47):36401–36409.
46. Inagaki T, et al. (2007) Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* 5(6):415–425.
47. Badman MK, et al. (2007) Hepatic fibroblast growth factor 21 is regulated by PPAR-alpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab* 5(6):426–437.
48. Lundåsen T, et al. (2007) PPARalpha is a key regulator of hepatic FGF21. *Biochem Biophys Res Commun* 360(2):437–440.
49. Purushotham A, et al. (2009) Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* 9(4): 327–338.
50. Potthoff MJ, et al. (2009) FGF21 induces PGC-1alpha and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. *Proc Natl Acad Sci USA* 106(26):10853–10858.
51. Bookout AL, et al. (2013) FGF21 regulates metabolism and circadian behavior by acting on the nervous system. *Nat Med* 19(9):1147–1152.
52. Emanuelli B, et al. (2014) Interplay between FGF21 and insulin action in the liver regulates metabolism. *J Clin Invest* 124(2):515–527.
53. Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115(5):577–590.
54. Choo AY, et al. (2010) Glucose addiction of TSC null cells is caused by failed mTORC1-dependent balancing of metabolic demand with supply. *Mol Cell* 38(4):487–499.
55. Durán RV, et al. (2012) Glutaminolysis activates Rag-mTORC1 signaling. *Mol Cell* 47(3): 349–358.
56. Csibi A, et al. (2013) The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4. *Cell* 153(4):840–854.
57. Fischer JE, Chance WT (1990) Total parenteral nutrition, glutamine, and tumor growth. *JPEN J Parenter Enteral Nutr* 14(Suppl 4):86S–89S.
58. Souba WW (1993) Glutamine and cancer. *Ann Surg* 218(6):715–728.
59. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* 7(1):11–20.
60. Chong-Kopera H, et al. (2006) TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase. *J Biol Chem* 281(13):8313–8316.
61. Meikle L, et al. (2007) A mouse model of tuberous sclerosis: Neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. *J Neurosci* 27(21):5546–5558.
62. Um SH, et al. (2004) Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431(7005):200–205.
63. Ueno M, et al. (2005) Regulation of insulin signalling by hyperinsulinaemia: Role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. *Diabetologia* 48(3): 506–518.
64. Shah OJ, Wang Z, Hunter T (2004) Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr Biol* 14(18):1650–1656.
65. Ozcan U, et al. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306(5695):457–461.
66. Appenzeller-Herzog C, Hall MN (2012) Bidirectional crosstalk between endoplasmic reticulum stress and mTOR signaling. *Trends Cell Biol* 22(5):274–282.
67. Yecies JL, et al. (2011) Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab* 14(1):21–32.
68. Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM (2010) mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* 468(7327): 1100–1104.
69. Kenerson HL, Yeh MM, Yeung RS (2011) Tuberous sclerosis complex-1 deficiency attenuates diet-induced hepatic lipid accumulation. *PLoS ONE* 6(3):e18075.
70. Preitner N, et al. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110(2):251–260.
71. Cho H, et al. (2012) Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. *Nature* 485(7396):123–127.
72. Asher G, et al. (2010) Poly(ADP-ribose) polymerase 1 participates in the phase entrainment of circadian clocks to feeding. *Cell* 142(6):943–953.
73. Feng D, et al. (2011) A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. *Science* 331(6022):1315–1319.
74. Yoon JC, et al. (2001) Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 413(6852):131–138.
75. Puigserver P, et al. (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* 423(6939):550–555.
76. Rhee J, et al. (2003) Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): Requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. *Proc Natl Acad Sci USA* 100(7):4012–4017.
77. Liu C, Li S, Liu T, Borjigin J, Lin JD (2007) Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature* 447(7143):477–481.
78. Leone TC, et al. (2005) PGC-1alpha deficiency causes multi-system energy metabolic derangements: Muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol* 3(4):e101.
79. Vega RB, Huss JM, Kelly DP (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 20(5):1868–1876.
80. Koo SH, et al. (2004) PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. *Nat Med* 10(5):530–534.
81. Schaap FG, Kremer AE, Lamers WH, Jansen PL, Gaemers IC (2013) Fibroblast growth factor 21 is induced by endoplasmic reticulum stress. *Biochimie* 95(4):692–699.
82. De Sousa-Coelho AL, Marrero PF, Haro D (2012) Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation. *Biochem J* 443(1): 165–171.
83. Kim KH, et al. (2013) Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine. *Nat Med* 19(1):83–92.
84. Kang YJ, Lu MK, Guan KL (2011) The TSC1 and TSC2 tumor suppressors are required for proper ER stress response and protect cells from ER stress-induced apoptosis. *Cell Death Differ* 18(1):133–144.
85. Yang C, et al. (2013) Activation of Liver FGF21 in hepatocarcinogenesis and during hepatic stress. *BMC Gastroenterol* 13:67.
86. Kim SG, et al. (2013) Metabolic stress controls mTORC1 lysosomal localization and dimerization by regulating the TTT-RUVBL1/2 complex. *Mol Cell* 49(1):172–185.
87. van der Vos KE, et al. (2012) Modulation of glutamine metabolism by the PI(3)K-PKB-FOXO network regulates autophagy. *Nat Cell Biol* 14(8):829–837.
88. Bentzinger CF, et al. (2008) Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell Metab* 8(5): 411–424.
89. Cunningham JT, et al. (2007) mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 450(7170):736–740.
90. Estall JL, et al. (2009) PGC-1alpha negatively regulates hepatic FGF21 expression by modulating the heme/Rev-Erb(alpha) axis. *Proc Natl Acad Sci USA* 106(52): 22510–22515.
91. Wang Y, Solt LA, Burris TP (2010) Regulation of FGF21 expression and secretion by retinoic acid receptor-related orphan receptor alpha. *J Biol Chem* 285(21): 15668–15673.
92. Menon S, et al. (2012) Chronic activation of mTOR complex 1 is sufficient to cause hepatocellular carcinoma in mice. *Sci Signal* 5(217):ra24.

Available online at www.sciencedirect.com
SciVerse ScienceDirect
Current Opinion in Genetics & Development

mTOR in aging, metabolism, and cancer

Marion Cornu[1], Verena Albert[1] and Michael N Hall

The target of rapamycin (TOR) is a highly conserved serine/threonine kinase that is part of two structurally and functionally distinct complexes, TORC1 and TORC2. In multicellular organisms, TOR regulates cell growth and metabolism in response to nutrients, growth factors and cellular energy. Deregulation of TOR signaling alters whole body metabolism and causes age-related disease. This review describes the most recent advances in TOR signaling with a particular focus on mammalian TOR (mTOR) in metabolic tissues vis-a-vis aging, obesity, type 2 diabetes, and cancer.

Address
Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Corresponding author: Hall, Michael N (m.hall@unibas.ch)
[1] These authors contributed equally to this work.

Current Opinion in Genetics & Development 2013, **23**:53–62
This review comes from a themed issue on **Cancer genomics**
Edited by **Nahum Sonenberg** and **Nissim Hay**
For a complete overview see the Issue and the Editorial
Available online 11th Jan 2013
0959-437X/$ – see front matter,
http://dx.doi.org/10.1016/j.gde.2012.12.005

Introduction

Target of rapamycin (TOR) is a conserved serine/threonine kinase that regulates cell growth, aging and metabolism, from yeast to human [1–5]. TOR is found in two structurally and functionally distinct complexes termed TOR complex 1 (TORC1) and TORC2 (Figures 1 and 2). The immunosuppressive macrolide rapamycin inhibits TORC1 activity. In metazoans, TORC1 controls growth-related processes such as ribosome biogenesis, protein synthesis, transcription, nutrient uptake and autophagy in response to nutrients, growth factors, and cellular energy status. The best-characterized substrates of TORC1 are 4E-BP and S6K via which mammalian TORC1 (mTORC1) controls protein synthesis. The core components of mTORC1 are mTOR, raptor and mLST8. mTORC2 is activated by growth factors alone, via PI3K-dependent ribosome association [6••,7••]. The commonly described substrates of TORC2 are AGC kinase family members such as Akt, SGK, and PKCα in mammals [8]. The core components of mTORC2 are mTOR, rictor, mSIN1 and mLST8.

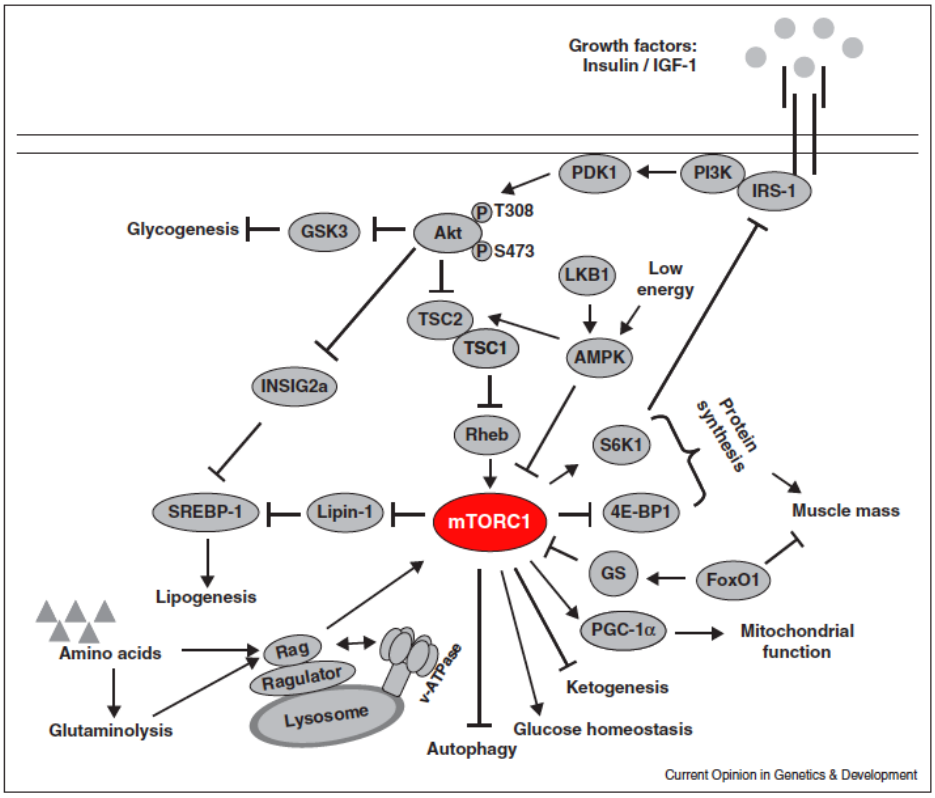
mTOR plays a particularly important role in metabolic organs — such as the liver, muscle, and adipose tissue — to regulate whole body energy homeostasis. Thus, deregulation of mTOR signaling leads to metabolic disorders, such as obesity and type 2 diabetes, and cancer, that is, some of the most common causes of death in Western society. Furthermore, consistent with its role as a nutrient and growth factor sensor, decreased mTOR signaling reduces aging and thereby extends lifespan. Importantly, aging is a major risk factor for the development of cancer and metabolic disorders. Thus, mTOR underlies both aging and age-related diseases, suggesting that insight in mTOR signaling may provide a means to counter both aging and age-related disease by a single 'treatment'. In other words, an understanding of mTOR signaling may allow one to collectively 'treat' age-related diseases by delaying aging. Here, we review the major recent findings on mTOR signaling in different metabolic organs and how this may affect aging and age-related disease.

TOR in aging

Aging is defined as an accumulation of cellular damage over time, promoting disease and death. Genetic or pharmacological inhibition of TORC1 signaling extends lifespan in yeast, worms, flies and mice [9,10•,11–19]. Importantly, rapamycin delays the onset of age-related disease and extends lifespan even in old mice [13••,15]. When started at a young age, rapamycin also delays decline in cognitive function [20]. Another intervention that slows the aging process is dietary restriction (DR) — a reduction in nutrient intake without malnutrition. DR prolongs lifespan in yeast, worms, flies, rodents, and possibly primates [21–23]. In mammals, DR also retards the onset of age-related disease. At the molecular level, the life-extending effects of DR appear to be due largely to inhibition of TOR, as suggested by the findings that TORC1 inhibition mimics starvation and DR does not further extend lifespan in yeast and flies with defective TORC1 signaling [17,19]. Moreover, S6K1 knockout mice are long-lived and display a phenotype similar to that observed upon DR [24].

The TORC1 substrate S6K (Sch9 in yeast) seems to have a pivotal role in regulating lifespan since S6K inhibition extends lifespan in yeast [17,25], worms [26–29], flies [19], and mice [24]. Furthermore, overexpression of a constitutively active form of S6K in *D. melanogaster* renders flies resistant to lifespan extension by rapamycin [11]. 4E-BP, the other well-characterized downstream target of TORC1, also mediates protective effects of DR and rapamycin treatment in flies [11,30]. Consistent with a role of the translation regulators S6K and 4E-BP in modulating lifespan, reduced protein synthesis also extends lifespan in many species [26,27,31–33]. Thus,

Figure 1



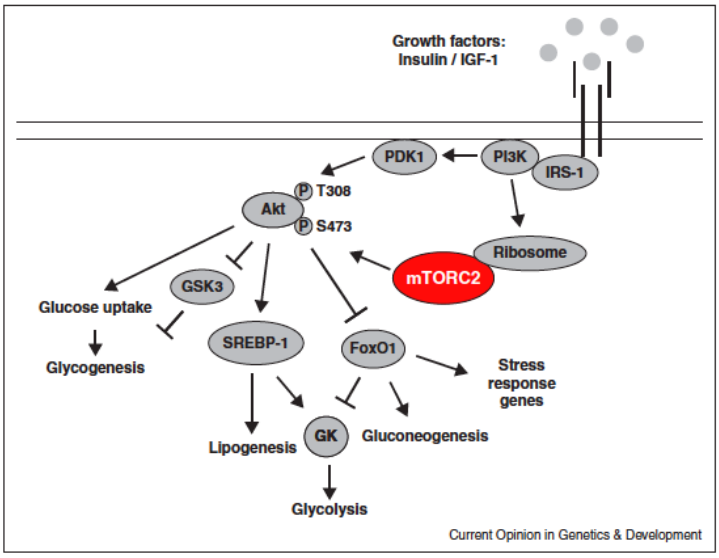
mTORC1 is activated by growth factors, amino acids, and cellular energy status to regulate protein synthesis, autophagy, mitochondrial function, lipogenesis, ketogenesis, and glucose homeostasis.

TORC1 appears to control aging via S6K and 4E-BP and ultimately the regulation of protein synthesis. Importantly, activation of 4E-BP also leads to activation of stress responsive genes, such as FoxO and Nrf, and genes encoding the mitochondrial electron transport chain (mETC) [10•,27,30,34–37]. Upregulation of stress genes exerts a positive effect on lifespan by protecting cells and tissues from age-related damage [35]. Hence, TORC1 may also control aging via modulation of stress responsive genes downstream of 4E-BP.

Autophagy has emerged as another downstream process via which TORC1 modulates aging [19,38]. Mice with a brain-specific knockout of the autophagy gene *Atg5* or *Atg7* have shorter lifespan and suffer from an accelerated form of age-related neuronal degeneration [39,40]. Autophagy also acts as a tumor suppressor. The oncogene BCL-2 binds beclin-1 and thereby suppresses autophagy and promotes tumorigenesis [41]. Thus, at least part of the lifespan extending effect of autophagy may be due to its role in cancer suppression.

The role of TORC2 in aging is less clear. Extension of lifespan upon TORC2 inactivation has been demonstrated in *C. elegans* [10•,42•]. The finding that TORC2 inhibition can increase lifespan raises an important question regarding the effects of rapamycin on aging. Is the extension of lifespan by rapamycin due to reduced TORC1, TORC2, or both? Chronic rapamycin treatment can also disrupt mTORC2 in certain cell lines [43]. Furthermore, chronic rapamycin treatment extends lifespan despite impairing glucose homeostasis in mice [44••], rats [45,46], and humans [47]. This raises the additional question, is the effect of rapamycin on glucose homeostasis due to mTORC1 or mTORC2 inhibition? Two recent studies in mice suggest that the diabetic phenotype observed upon prolonged rapamycin treatment is due to mTORC2 inactivation [44••,48••]. Adult

Figure 2



mTORC2 is activated by growth factors to regulate glycolysis, gluconeogenesis, glycogenesis, and lipogenesis.

mice with a liver-specific [48••] or an induced whole-body deletion of *rictor* [44••] exhibit glucose intolerance, and, as shown in the latter report, this phenotype is not exacerbated by rapamycin treatment. Unfortunately, neither study investigated whether genetic ablation of mTORC2 signaling alone is sufficient to modulate lifespan. However, reduction solely of mTORC1 signaling is able to increase lifespan. Female mice carrying a single copy of *mTOR* and *mLST8* are long lived. Molecular analysis of the $mtor^{+/-}$ $mlst8^{+/-}$ mice revealed that mTORC1 signaling was reduced whereas mTORC2 signaling was intact [44••]. This finding is unexpected because mLST8 and mTOR are found in both mTOR complexes, and because *LST8* deletion was shown previously to inactivate TORC2 signaling without affecting TORC1 in mice [49], flies [50], and yeast [51]. Accounting for the inverted phenotype, Lamming *et al.* [44••] report that raptor binding to mTOR is reduced while rictor binding to mTOR is unaffected in $mtor^{+/-}$ $mlst8^{+/-}$ mice compared to control animals. Surprisingly, no effect on aging was observed in mice carrying only one copy of *mTOR*, *raptor*, or both *mTOR* and *raptor*.

Is reduction of TOR activity in a specific tissue(s), as opposed to the whole organism, sufficient to extend lifespan? Recent findings suggest that this is indeed the case. Worms with an intestine-specific inactivation TORC1 or TORC2 live longer [10•]. The worm intestine corresponds to the gut, adipose tissue and liver in mammals. Flies with a fat body-specific ablation of TORC1 signaling are also long lived [52]. The fly fat body corresponds to adipose tissue and the liver. Mice with an adipose tissue-specific deletion of *raptor* are lean and protected against diet-induced obesity, although it remains to be determined whether such mice live longer [53•]. In summary, it appears that reducing TOR signaling specifically in a metabolic tissue may be sufficient to extend lifespan.

It is well established that reduced signaling through the insulin/IGF-1 signaling (IIS) pathway also extends lifespan [reviewed in 54]. Tissue-specific modulation of the IIS pathway is sufficient to delay aging. Adipose-specific insulin receptor knockout mice exhibit increased lifespan, reduced adiposity, and are protected against age-related obesity [55]. Interestingly, a deletion of the insulin receptor in any other important metabolic organ, such as the liver [56], pancreas [57], or muscle [58], results in a diabetic phenotype without any beneficial effect on aging. Similarly, tissue-specific modulation of mTORC1 or mTORC2 signaling reveals that each complex exerts different functions in different organs with regard to whole body glucose and lipid homeostasis (discussed in the following section). These observations complicate the development of anti-aging drugs targeting the mTOR and IIS pathways.

Inhibition of the IIS pathway activates the transcription factor FoxO, and many of the lifespan extending effects of IIS inhibition are indeed mediated by FoxO [59]. FoxO also acts as a tumor suppressor [60,61]. Interestingly, a recent study in mammalian cells and *C. elegans* demonstrated that FoxO/DAF-16 activates expression of glutamine synthetase (GS) and increased GS expression in turn inhibits TORC1 activity [62]. In agreement with this finding, another recent report demonstrated that glutaminolysis (the de-amination of glutamine to form α-ketoglutarate) activates mTORC1 [63••]. Furthermore, in flies and mammals FoxO blocks TORC1 signaling by inducing expression of sestrins which leads to activation of AMPK, a negative regulator of TORC1 signaling [64–66]. In worms, DAF-16 negatively regulates *raptor/daf-15* transcription [67]. These findings suggest that FoxO may exert some of its positive effects on lifespan and tumor suppression via inhibition of TORC1 signaling.

mTOR in metabolic tissues

mTOR signaling is found in all tissues, but is probably particularly important in metabolic tissues. Metabolic organs, such as the liver, muscle and adipose tissue, are particularly sensitive to nutrients, insulin/IGF-1, and energy — the three inputs that control mTOR. Liver, muscle and adipose tissue, in turn, control whole body glucose and lipid homeostasis. Below we review recent studies on the regulation of glucose and lipid homeostasis by mTOR in metabolic tissues.

mTOR in the liver

Upon fasting, the liver produces glucose via glycogen breakdown (glycogenolysis) or via glucose synthesis (gluconeogenesis), to prevent hypoglycemia. Upon feeding, the liver reduces blood glucose levels via consumption (glycolysis) or via conversion of glucose to glycogen (glycogenesis) or triglyceride (lipogenesis). Genetically modified mice with defective mTOR signaling in the liver are glucose intolerant, hyperglycemic, hyperinsulinemic and display decreased glycogen content [44••,48••,68•,69••,70••], indicating that hepatic mTOR plays a major role in glucose homeostasis. Furthermore, the above defects are similar to those observed in patients with type 2 diabetes, suggesting that defective mTOR signaling in the liver accounts, at least partly, for the pathophysiology of type 2 diabetes.

Lipogenesis is activated via the transcription factor sterol regulatory element-binding protein (SREBP) [71–73]. As first demonstrated in retinal pigment epithelial cells and mouse embryonic fibroblasts (MEFs), mTORC1 mediates maturation of SREBP-1 in an S6K1-dependent manner to stimulate *de novo* lipid synthesis [74,75]. However, as shown in primary hepatocytes, mTORC1 stimulates SREBP-1 expression in an S6K-independent manner [76]. These data suggest that mTORC1 activates SREBP expression and maturation, but via two separate effector pathways. The S6K-independent pathway involves the mTORC1 substrate phosphatidic acid phosphatase lipin-1, a negative regulator of SREBP-1 activity [77•]. In response to nutrients and growth factors, mTORC1 directly phosphorylates lipin-1. This prevents translocation of lipin-1 into the nucleus, thereby allowing SREBP transcriptional activity. Although it is well established that mTORC1 is required to activate SREBP-1 and lipid synthesis in cultured cells, the role of mTORC1 in lipogenesis *in vivo* is less clear. Liver-specific mTORC1 deficient (*raptor* knockout) mice display decreased hepatic triglyceride content and a reduction in plasma cholesterol levels only when fed a high fat diet [77•]. Thus, mTORC1 signaling appears to be necessary for hepatic triglyceride accumulation *in vivo* only under pathological conditions.

Patients with type 2 diabetes exhibit 'selective hepatic insulin resistance'. This is a state in which insulin fails to inhibit hepatic glucose production yet paradoxically maintains lipogenesis, resulting in hyperglycemia and hyperlipidemia [78]. However, humans with mutations in the insulin receptor gene or liver-specific insulin receptor knockout mice exhibit hyperglycemia and hypolipidemia — a state referred to as 'total hepatic insulin resistance' in which insulin is unable to suppress hepatic glucose production or to stimulate lipogenesis [56,79,80]. It was suggested that selective hepatic insulin resistance might be due to nutrient activated mTORC1 even in the absence of upstream, insulin-stimulated Akt activity [76,81]. However, three independent studies have shown that liver-specific *tsc1* knockout mice (L*Tsc1*KO), in which mTORC1 is ectopically activated, are protected against age-induced and diet-induced hepatic steatosis [69••,70••,82••]. Yecies *et al.* [70••] demonstrated that protection against hepatic lipid accumulation in L*Tsc1*KO mice is due to attenuation of Akt signaling, as restoration of Akt2 (the main hepatic isoform of Akt) signaling restores lipogenesis. This suggests that Akt and mTORC1 are independently necessary for lipogenesis. Decreased Akt signaling in L*Tsc1*KO mice is due to the well-known mTORC1-mediated negative feedback loop [70••]. Yecies *et al.* [70••] propose that Akt is required to prevent expression of *Insig2a* encoding an SREBP inhibitor. mTORC1 is required for a separate step in the activation of SREBP, as described further above. Thus, both Akt and mTORC1 are required for lipogenesis, and the molecular basis of selective hepatic insulin resistance remains to be determined. However, complicating matters, Kenerson *et al.* [69••] reported that mTORC1 is not necessary for hepatic lipid accumulation, since rapamycin treatment fails to prevent high-fat diet or *Pten* deletion-induced hepatic steatosis.

mTORC2 is also insulin-stimulated and is required in the liver for lipid and glucose homeostasis. Liver-specific *rictor* knockout (LiRiKO) mice are hypolipidemic and

hyperglycemic [44••,48••,68•], reflecting a state of total hepatic insulin resistance. This is due to loss of Akt Ser473 phosphorylation [48••]. Similar to L*Tsc1*KO mice, LiRiKO mice show reduced SREBP-1c activity. Again, restoration of Akt signaling suppressed the defects in SREBP-1c activity and *de novo* lipogenesis [48••]. Defects in SREBP-1c activity and hepatic lipogenesis in LiRiKO mice, where mTORC2 but not mTORC1 is impaired, suggest that Akt regulates SREBP-1c at least partly independently of mTORC1. Interestingly, *Insig2a* regulation was not changed in the liver of LiRiKO mice, indicating that Akt Ser473 phosphorylation is not necessary for *Insig2a* inhibition. In conclusion, mTORC1, mTORC2, and Akt are required for lipogenesis in the liver.

Hepatic mTORC2 controls glucose homeostasis via activation of glycolysis and inhibition of gluconeogenesis [48••]. mTORC2 stimulates glycolysis through activation of glucokinase and the transcription factor ChREBP. mTORC2 inhibits gluconeogenesis by inhibiting nuclear accumulation of FoxO1. The regulation of at least glucokinase and FoxO1 are via phosphorylation of Akt Ser473. These findings demonstrate that in the liver mTORC2 tightly regulates Akt to control glucose and lipid homeostasis and thereby whole body metabolism. A defect in hepatic mTORC2 signaling may contribute to the development of diabetes.

mTOR in muscle

mTOR or *raptor* knockout mice have been generated to determine the *in vivo* function of mTORC1 signaling in skeletal and cardiac muscle. Skeletal muscle-specific knockout mice develop progressive muscle dystrophy and display decreased oxidative capacity and increased glycogen content [83,84••]. Skeletal muscle of S6K1 deficient mice becomes atrophic and accumulates glycogen, suggesting that mTORC1 controls muscle mass and physiology through at least S6K1 [85,86]. Muscle of S6K1 deficient mice display increased rather than decreased mitochondrial activity, suggesting that mTORC1 may regulate mitochondrial oxidative capacity through a substrate other than S6K1 [86]. Cardiac-specific *mTOR* or *raptor* knockout mice develop dilated cardiomyopathy due to loss of 4E-BP1 inhibition and thus reduced protein synthesis [87,88].

The increased glycogen accumulation observed in skeletal muscle-specific *mTOR* or *raptor* knockout mice is mediated by Akt hyperactivation due to the loss of the negative feedback loop [83,84••]. Despite Akt hyperactivation, muscle-specific *raptor* knockout mice are slightly glucose intolerant. This is unexpected and thus requires further study since Akt activates glycolysis and glucose uptake. The decrease in mitochondrial oxidative capacity observed in the *raptor* knockout mice is due to a reduction in PGC-1α, since the defect is suppressed by restoration of PGC-1α expression [89].

Skeletal muscle-specific *rictor* knockout mice display little-to-no phenotype despite reduced Akt Ser473 phosphorylation [84••,90]. The mice are slightly glucose intolerant, probably due to loss of Akt-medited AS160 phosphorylation. AS160 is a major Akt substrate required for insulin-stimulated translocation of the glucose transporter GLUT4 to the plasma membrane [90].

mTOR in adipose tissue

Excessive white adipose tissue (WAT) accumulation (obesity) increases the risk of developing metabolic disorders such as insulin resistance, type 2 diabetes, cardiovascular diseases and cancer. The role of mTOR signaling in adipose tissue has been studied *in vitro* and *in vivo*. Rapamycin treatment inhibits *in vitro* differentiation of mouse and human pre-adipocytes [53•,91–95]. Moreover, mTORC1 inhibition in cultured cells decreases expression of the adipogenic transcription factors peroxisome proliferators-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein-α (C/EBP-α) [53•,92,94,95]. Conversely, hyperactivation of mTORC1 by *Tsc2* deletion increases adipogenesis by enhancing PPAR-γ expression [96]. Thus, mTORC1 mediates adipocyte differentiation and maintenance in isolated cells via activation of PPAR-γ and C/EBP-α. Adipose-specific raptor knockout ($raptor^{ad-/-}$) mice are lean and protected against diet-induced obesity. The reduced weight is due to smaller and fewer adipocytes [53•]. This suggests that mTORC1 also plays an important role in adipocyte metabolism *in vivo*. However, contrary to what was observed in mTORC1-deficient cultured cells, $raptor^{ad-/-}$ mice display normal levels of PPAR-γ and C/EBP-α in epididymal WAT, suggesting that *in vivo* other factors may be involved in the regulation of PPAR-γ and C/EBP-α expression. The leanness of $raptor^{ad-/-}$ mice is due to enhanced energy expenditure resulting from UCP1-mediated mitochondrial uncoupling in WAT [53•]. Consistent with the phenotype observed in $raptor^{ad-/-}$ mice, full-body S6K1 knockout mice are also lean, protected against age-induced and diet-induced obesity. Conversely, mice lacking 4E-BP1 and 4E-BP2 exhibit increased sensitivity to diet-induced obesity with reduced energy expenditure [97]. Triple knockout mice lacking S6K1 and the two 4E-BPs resemble the *raptor* or *S6K1* knockout mice, suggesting that mTORC1 controls adipose metabolism mainly via S6K1 [98]. Altogether, the above studies demonstrate that mTORC1 is an important regulator of adipose metabolism and thereby of whole body homeostasis.

Interestingly, adipose-specific rictor knockout ($rictor^{ad-/-}$) mice display an increase in body size due to an increase in lean mass while fat mass is largely unaffected [99,100]. This phenotype can be explained by the observation that mTORC2 in WAT negatively regulates IGF-1 and insulin production by the liver and pancreas, respectively, thereby regulating systemic growth and glucose and lipid

metabolism [100]. Adipose mTORC2-mediated regulation of IGF-1 and insulin may be due to a negative feedback endocrine loop, since mTORC2 is itself activated by these hormones. The factor(s) that might signal from adipose to the liver and pancreas as part of such a loop remain to be identified. The purpose of such a loop would be to maintain hormone homeostasis.

mTOR in cancer

mTOR is frequently activated in human cancers [3,101]. Accumulating evidence suggests that aberrant regulation of both cell growth and metabolism significantly contribute to cancer development and progression [102]. The notion of causal changes in metabolism during cancer development is supported by the observation that obesity and diabetes are risk factors for cancer and that diet can affect tumor growth [103–107]. For example, hepatic steatosis often leads to hepatocellular carcinoma (HCC) [108]. Also, metformin, the most commonly prescribed anti-diabetic drug, reduces the incidence of cancer [109,110]. As discussed above, mTOR signaling plays a central role in metabolism. The fact that an mTOR signaling defect can cause both metabolic disorders and cancer suggests that mTOR links cancer development and metabolism. This is supported by the observation that metformin inhibits mTORC1 signaling, via activation of AMPK and REDD1 and a Rag GTPase-sensitive mechanism, in addition to reducing cancer [111–113]. A recent study demonstrated that metformin's anti-proliferative activity is due to a 4E-BP-dependent decrease in translation [114]. mTORC1, via inhibition of 4E-BP, appears to activate translation of pro-oncogenic mRNAs with 5′ terminal oligopyrimidine (5′TOP) motifs [115,116]. These data suggest that regulation of 4E-BP by mTORC1 plays a particularly important role in cell proliferation and cancer development. Further supporting this hypothesis, rapamycin and its analogs (rapalogs), which only partly inhibit mTOR-dependent phosphorylation of 4E-BP, are only partly successful as a cancer treatment [117]. On the other hand, ATP competitive mTOR inhibitors that fully inhibit mTOR [110] and therefore also fully inhibit 4E-BP phosphorylation have stronger antitumor effects [118]. Dowling *et al.* propose that mTORC1 controls cell proliferation exclusively via 4E-BP while it regulates cell growth via S6K [119]. This would mean that in mammalian cells control of cell size and cell cycle progression are independent of each other. However, how proliferation can occur independently of cell growth remains to be clarified. Further evidence suggesting that mTOR links metabolism and cancer is provided by a recent study demonstrating that L*Tsc*1KO mice with hyperactive mTORC1 signaling display metabolic abnormalities, including defects in glucose and lipid homeostasis, and subsequently develop HCC [69••,70••,120•]. Interestingly, liver-specific *Pten* knockout mice, which also exhibit increased mTORC1 activity, develop hepatic steatosis before the onset of liver cancer [121]. The tumor suppressor PTEN is also a negative regulator of mTORC2, and mTORC2 is required for the development of prostate cancer induced by *Pten* loss [122]. The association of mTORC2 with ribosomes is enhanced in PTEN-deficient melanoma cell lines and this leads to enhanced Akt phosphorylation [6••]. However, the mechanism by which mTORC2 is activated upon interaction with ribosomes still needs to be clarified.

Glutaminolysis provides an interesting mTOR-related link between metabolism and cancer. Highly proliferating cancer cells are often glutamine-addicted, and tumor growth correlates with the activity of glutaminase (GLS), the enzyme that catalyzes the first step of glutaminolysis [123,124]. Conversely, inhibition of GLS blocks cancer development and slows growth in certain gliomas [125,126]. Duran *et al.* [63••] recently demonstrated that glutaminolysis also activates mTORC1, thereby promoting cell growth and inhibiting autophagy [63••]. These findings suggest that glutaminolysis promotes cancer, at least partly, via mTORC1 activation. Targeting both glutaminolysis and mTORC1 may be a strategy for treatment of glutamine-addicted tumors.

Conclusions

This review emphasizes the importance of mTOR signaling in aging, whole body metabolism, and cancer. Tissue-specific mTORC1 and mTORC2 deletions have revealed that each of the two complexes has different roles in different organs with regard to whole body glucose and lipid homeostasis. For example impaired mTORC2 signaling in the liver and muscle leads to a diabetic phenotype whereas mTORC2 deletion in adipose tissue does not cause diabetes. Similarly, deletion of mTORC1 signaling in muscle but not in adipose tissue or liver leads to glucose intolerance. Thus, the development of treatments that target mTOR signaling to delay aging or to treat metabolic disorders and cancer will require understanding tissue-specific mTOR signaling. Even though rapamycin has been shown to increase lifespan and to protect against cancer, side effects such as immunosuppression or diabetes may limit rapamycin's usefulness as a potential longevity drug.

Acknowledgements

We acknowledge support from the Swiss National Science Foundation, the Swiss Cancer League, the Louis-Jeantet Foundation, the SFD-ALFEDIAM (MC), the Werner Siemens Foundation (VA) and the Canton of Basel.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Wullschleger S, Loewith R, Hall MN: **TOR signaling in growth and metabolism**. *Cell* 2006, **124**:471-484.

2. Hansen M, Kapahi P: **TOR signaling and aging**. In *The Enzymes: Structure, Function and Regulation of TOR Complexes from Yeasts*

to Mammals, vol 28. Edited by Hall MN, Tamanoi F. Academic Press, Elsevier Inc.; 2010:279-299.

3. Laplante M, Sabatini DM: **mTOR signaling in growth control and disease**. *Cell* 2012, **149**:274-293.

4. Loewith R, Hall MN: **Target of rapamycin (TOR) in nutrient signaling and growth control**. *Genetics* 2011, **189**:1177-1201.

5. Kim J, Guan KL: **Amino acid signaling in TOR activation**. *Annu Rev Biochem* 2011, **80**:1001-1032.

6. •• Zinzalla V, Stracka D, Oppliger W, Hall MN: **Activation of mTORC2 by association with the ribosome**. *Cell* 2011, **144**:757-768.
This study together with [7••] shows that mTORC2 signaling is regulated by ribosome association

7. •• Oh WJ, Wu CC, Kim SJ, Facchinetti V, Julien LA, Finlan M, Roux PP, Su B, Jacinto E: **mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide**. *EMBO J* 2010, **29**: 3939-3951.
This study together with [6••] shows that mTORC2 is associated with the ribosome

8. Cybulski N, Hall MN: **TOR complex 2: a signaling pathway of its own**. *Trends Biochem Sci* 2009, **34**:620-627.

9. Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S: **Extension of chronological life span in yeast by decreased TOR pathway signaling**. *Genes Dev* 2006, **20**:174-184.

10. • Robida-Stubbs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD, Neumann-Haefelin E, Sabatini DM, Blackwell TK: **TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO**. *Cell Metab* 2012, **15**:713-724.
This study together with [42•] shows that TORC2 signaling can influence lifespan in *C. elegans*

11. Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L: **Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster**. *Cell Metab* 2010, **11**: 35-46.

12. Chen D, Thomas EL, Kapahi P: **HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in *Caenorhabditis elegans***. *PLoS Genet* 2009, **5**:e1000486.

13. •• Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS *et al.*: **Rapamycin fed late in life extends lifespan in genetically heterogeneous mice**. *Nature* 2009, **460**:392-395.
This study demonstrates that rapamycin extends lifespan even when fed late in life

14. Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP, Blagosklonny MV: **Rapamycin extends maximal lifespan in cancer-prone mice**. *Am J Pathol* 2010, **176**: 2092-2097.

15. Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF *et al.*: **Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice**. *J Gerontol A Biol Sci Med Sci* 2011, **66**:191-201.

16. Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK *et al.*: **Rapamycin slows aging in mice**. *Aging Cell* 2012, **11**:675-682.

17. Kaeberlein M, Powers RW 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK: **Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients**. *Science* 2005, **310**:1193-1196.

18. Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F: **Genetics: influence of TOR kinase on lifespan in *C. elegans***. *Nature* 2003, **426**:620.

19. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S: **Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway**. *Curr Biol* 2004, **14**:885-890.

20. Majumder S, Caccamo A, Medina DX, Benavides AD, Javors MA, Kraig E, Strong R, Richardson A, Oddo S: **Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1beta and enhancing NMDA signaling**. *Aging Cell* 2012, **11**:326-335.

21. Masoro EJ: **Subfield history: caloric restriction, slowing aging, and extending life**. *Sci Aging Knowledge Environ* 2003, **2003**:RE2.

22. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW *et al.*: **Caloric restriction delays disease onset and mortality in rhesus monkeys**. *Science* 2009, **325**:201-204.

23. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, Longo DL, Allison DB, Young JE, Bryant M *et al.*: **Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study**. *Nature* 2012, **489**:318-321.

24. Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F *et al.*: **Ribosomal protein S6 kinase 1 signaling regulates mammalian life span**. *Science* 2009, **326**:140-144.

25. Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD: **Regulation of longevity and stress resistance by Sch9 in yeast**. *Science* 2001, **292**:288-290.

26. Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C: **Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans***. *Aging Cell* 2007, **6**:95-110.

27. Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ, Kapahi P: **Inhibition of mRNA translation extends lifespan in *Caenorhabditis elegans***. *Aging Cell* 2007, **6**:111-119.

28. Chen C, Liu Y, Liu Y, Zheng P: **mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells**. *Sci Signal* 2009, **2**:ra75.

29. Sheaffer KL, Updike DL, Mango SE: **The Target of Rapamycin pathway antagonizes pha-4/FoxA to control development and aging**. *Curr Biol* 2008, **18**:1355-1364.

30. Zid BM, Rogers AN, Katewa SD, Vargas MA, Kolipinski MC, Lu TA, Benzer S, Kapahi P: **4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila**. *Cell* 2009, **139**:149-160.

31. Henderson ST, Bonafe M, Johnson TE: **daf-16 protects the nematode *Caenorhabditis elegans* during food deprivation**. *J Gerontol A Biol Sci Med Sci* 2006, **61**:444-460.

32. Steffen KK, MacKay VL, Kerr EO, Tsuchiya M, Hu D, Fox LA, Dang N, Johnston ED, Oakes JA, Tchao BN *et al.*: **Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4**. *Cell* 2008, **133**:292-302.

33. Syntichaki P, Troulinaki K, Tavernarakis N: **Protein synthesis is a novel determinant of aging in *Caenorhabditis elegans***. *Ann N Y Acad Sci* 2007, **1119**:289-295.

34. Rogers AN, Chen D, McColl G, Czerwieniec G, Felkey K, Gibson BW, Hubbard A, Melov S, Lithgow GJ, Kapahi P: **Life span extension via eIF4G inhibition is mediated by posttranscriptional remodeling of stress response gene expression in *C. elegans***. *Cell Metab* 2011, **14**:55-66.

35. Wang J, Robida-Stubbs S, Tullet JM, Rual JF, Vidal M, Blackwell TK: **RNAi screening implicates a SKN-1-dependent transcriptional response in stress resistance and longevity deriving from translation inhibition**. *PLoS Genet* 2010:6.

36. Murphy CT: **The search for DAF-16/FOXO transcriptional targets: approaches and discoveries**. *Exp Gerontol* 2006, **41**:910-921.

37. Pan Y, Schroeder EA, Ocampo A, Barrientos A, Shadel GS: **Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling**. *Cell Metab* 2011, **13**:668-678.

38. Toth ML, Sigmond T, Borsos E, Barna J, Erdelyi P, Takacs-Vellai K, Orosz L, Kovacs AL, Csikos G, Sass M *et al.*: **Longevity pathways converge on autophagy genes to regulate life span in *Caenorhabditis elegans***. *Autophagy* 2008, **4**:330-338.

39. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E *et al.*: **Loss of**

autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 2006, **441**:880-884.

40. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H *et al.*: **Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice**. *Nature* 2006, **441**:885-889.

41. Pattingre S, Levine B: **Bcl-2 inhibition of autophagy: a new route to cancer?** *Cancer Res* 2006, **66**:2885-2888.

42. • Soukas AA, Kane EA, Carr CE, Melo JA, Ruvkun G: **Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans***. *Genes Dev* 2009, **23**:496-511.
This study together with [10•] shows that TORC2 signaling can influence lifespan in *C. elegans*

43. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM: **Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB**. *Mol Cell* 2006, **22**:159-168.

44. •• Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS *et al.*: **Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity**. *Science* 2012, **335**:1638-1643.
This study highlights that prolonged rapamycin treatment results in diabetes due to mTORC2 inhibition

45. Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N *et al.*: **mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes**. *Diabetes* 2008, **57**:945-957.

46. Houde VP, Brule S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A: **Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue**. *Diabetes* 2010, **59**:1338-1348.

47. Johnston O, Rose CL, Webster AC, Gill JS: **Sirolimus is associated with new-onset diabetes in kidney transplant recipients**. *J Am Soc Nephrol* 2008, **19**:1411-1418.

48. •• Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN: **Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c**. *Cell Metab* 2012, **15**:725-738.
This study together with [68] demonstrates that mTORC2 in the liver controls glucose and lipid homeostasis

49. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM: **Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1**. *Dev Cell* 2006, **11**:859-871.

50. Wang T, Blumhagen R, Lao U, Kuo Y, Edgar BA: **LST8 regulates cell growth via target-of-rapamycin complex 2 (TORC2)**. *Mol Cell Biol* 2012, **32**:2203-2213.

51. Pracheil T, Thornton J, Liu Z: **TORC2 signaling is antagonized by protein phosphatase 2A and the Far complex in Saccharomyces cerevisiae**. *Genetics* 2012, **190**:1325-1339.

52. Luong N, Davies CR, Wessells RJ, Graham SM, King MT, Veech R, Bodmer R, Oldham SM: **Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity**. *Cell Metab* 2006, **4**:133-142.

53. • Polak P, Cybulski N, Feige JN, Auwerx J, Ruegg MA, Hall MN: **Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration**. *Cell Metab* 2008, **8**:399-410.
This study demonstrates that mTORC1 in adipose tissue regulates whole body energy homeostasis

54. Kenyon CJ: **The genetics of ageing**. *Nature* 2010, **464**:504-512.

55. Bluher M, Kahn BB, Kahn CR: **Extended longevity in mice lacking the insulin receptor in adipose tissue**. *Science* 2003, **299**:572-574.

56. Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR: **Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction**. *Mol Cell* 2000, **6**:87-97.

57. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: **Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes**. *Cell* 1999, **96**:329-339.

58. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR: **A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance**. *Mol Cell* 1998, **2**:559-569.

59. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G: **The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans***. *Nature* 1997, **389**:994-999.

60. Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H *et al.*: **IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a**. *Cell* 2004, **117**:225-237.

61. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, Miao L, Tothova Z, Horner JW, Carrasco DR *et al.*: **FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis**. *Cell* 2007, **128**:309-323.

62. van der Vos KE, Eliasson P, Proikas-Cezanne T, Vervoort SJ, van Boxtel R, Putker M, van Zutphen IJ, Mauthe M, Zellmer S, Pals C *et al.*: **Modulation of glutamine metabolism by the PI(3)K-PKB-FOXO network regulates autophagy**. *Nat Cell Biol* 2012, **14**:829-837.

63. •• Duran RV, Oppliger W, Robitaille AM, Heiserich L, Skendaj R, Gottlieb E, Hall MN: **Glutaminolysis Activates Rag-mTORC1 Signaling**. *Mol Cell* 2012.
This study provides a molecular mechanism how the amino acid leucine is sensed by mTORC1 and this may explain glutamine addiction in cancer cells

64. Chen CC, Jeon SM, Bhaskar PT, Nogueira V, Sundararajan D, Tonic I, Park Y, Hay N: **FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor**. *Dev Cell* 2010, **18**:592-604.

65. Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R, Bier E *et al.*: **Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies**. *Science* 2010, **327**:1223-1228.

66. Budanov AV, Karin M: **p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling**. *Cell* 2008, **134**:451-460.

67. Jia K, Chen D, Riddle DL: **The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span**. *Development* 2004, **131**:3897-3906.

68. • Yuan M, Pino E, Wu L, Kacergis M, Soukas AA: **Identification of Akt-Independent Regulation of Hepatic Lipogenesis by Mammalian Target of Rapamycin (mTOR) Complex 2**. *J Biol Chem* 2012.
This study together with [48••] demonstrates that mTORC2 in the liver controls glucose and lipid homeostasis

69. •• Kenerson HL, Yeh MM, Yeung RS: **Tuberous sclerosis complex-1 deficiency attenuates diet-induced hepatic lipid accumulation**. *PLoS ONE* 2011, **6**:e18075.
This study, together with [70••,82••] shows that hepatic mTORC1 regulates lipid homeostasis

70. •• Yecies JL, Zhang HH, Menon S, Liu S, Yecies D, Lipovsky AI, Gorgun C, Kwiatkowski DJ, Hotamisligil GS, Lee CH *et al.*: **Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways**. *Cell Metab* 2011, **14**:21-32.
This study together with [69••,82••] shows that hepatic mTORC1 regulates lipid homeostasis due to attenuation of Akt signaling

71. Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K *et al.*: **Sterol**

regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem* 1999, **274**:35832-35839.

72. Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL: **Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes**. *Proc Natl Acad Sci U S A* 1999, **96**:13656-13661.

73. Foretz M, Guichard C, Ferre P, Foufelle F: **Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes**. *Proc Natl Acad Sci U S A* 1999, **96**:12737-12742.

74. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, Griffiths JR, Chung YL, Schulze A: **SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth**. *Cell Metab* 2008, **8**:224-236.

75. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma Q, Gorski R, Cleaver S *et al.*: **Activation of a metabolic gene regulatory network downstream of mTOR complex 1**. *Mol Cell* 2010, **39**:171-183.

76. Li S, Brown MS, Goldstein JL: **Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis**. *Proc Natl Acad Sci U S A* 2010, **107**:3441-3446.

77. • Peterson TR, Sengupta SS, Harris TE, Carmack AE, Kang SA, Balderas E, Guertin DA, Madden KL, Carpenter AE, Finck BN *et al.*: **mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway**. *Cell* 2011, **146**:408-420.
This study demonstrates that mTORC1 activates SREBP and thus hepatic lipogenesis in a rapamycin-independent manner

78. Brown MS, Goldstein JL: **Selective versus total insulin resistance: a pathogenic paradox**. *Cell Metab* 2008, **7**:95-96.

79. Semple RK, Sleigh A, Murgatroyd PR, Adams CA, Bluck L, Jackson S, Vottero A, Kanabar D, Charlton-Menys V, Durrington P *et al.*: **Postreceptor insulin resistance contributes to human dyslipidemia and hepatic steatosis**. *J Clin Invest* 2009, **119**:315-322.

80. Biddinger SB, Hernandez-Ono A, Rask-Madsen C, Haas JT, Aleman JO, Suzuki R, Scapa EF, Agarwal C, Carey MC, Stephanopoulos G *et al.*: **Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis**. *Cell Metab* 2008, **7**:125-134.

81. Laplante M, Sabatini DM: **mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis**. *Proc Natl Acad Sci U S A* 2010, **107**:3281-3282.

82. •• Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM: **mTORC1 controls fasting-induced ketogenesis and its modulation by ageing**. *Nature* 2010, **468**:1100-1104.
This study together with [69••,70••] shows that hepatic mTORC1 regulates lipid homeostasis. In particular, this study addresses the role of hepatic mTORC1 in ketogenesis

83. Risson V, Mazelin L, Roceri M, Sanchez H, Moncollin V, Corneloup C, Richard-Bulteau H, Vignaud A, Baas D, Defour A *et al.*: **Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy**. *J Cell Biol* 2009, **187**:859-874.

84. •• Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F, Xia J, Casanova E, Costa CF, Brink M *et al.*: **Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy**. *Cell Metab* 2008, **8**:411-424.
This study demonstrates that mTORC1 signaling in skeletal muscle regulates whole body energy homeostasis. In the muscle, mTORC1 controls oxidative capacity, glycogen content, and muscle mass

85. Ohanna M, Sobering AK, Lapointe T, Lorenzo L, Praud C, Petroulakis E, Sonenberg N, Kelly PA, Sotiropoulos A, Pende M: **Atrophy of S6K1(−/−) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control**. *Nat Cell Biol* 2005, **7**:286-294.

86. Aguilar V, Alliouachene S, Sotiropoulos A, Sobering A, Athea Y, Djouadi F, Miraux S, Thiaudiere E, Foretz M, Viollet B *et al.*: **S6 kinase deletion suppresses muscle growth adaptations to nutrient availability by activating AMP kinase**. *Cell Metab* 2007, **5**:476-487.

87. Zhang D, Contu R, Latronico MV, Zhang J, Zhang JL, Rizzi R, Catalucci D, Miyamoto S, Huang K, Ceci M *et al.*: **MTORC1 regulates cardiac function and myocyte survival through 4E-BP1 inhibition in mice**. *J Clin Invest* 2010, **120**:2805-2816.

88. Shende P, Plaisance I, Morandi C, Pellieux C, Berthonneche C, Zorzato F, Krishnan J, Lerch R, Hall MN, Rüegg MA *et al.*: **Cardiac raptor ablation impairs adaptive hypertrophy, alters metabolic gene expression, and causes heart failure in mice**. *Circulation* 2011, **123**:1073-1082.

89. Romanino K, Mazelin L, Albert V, Conjard-Duplany A, Lin S, Bentzinger CF, Handschin C, Puigserver P, Zorzato F, Schaeffer L *et al.*: **Myopathy caused by mammalian target of rapamycin complex 1 (mTORC1) inactivation is not reversed by restoring mitochondrial function**. *Proc Natl Acad Sci U S A* 2011, **108**:20808-20813.

90. Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, Lawrence JC Jr: **Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity**. *Mol Cell Biol* 2008, **28**:61-70.

91. Bell A, Grunder L, Sorisky A: **Rapamycin inhibits human adipocyte differentiation in primary culture**. *Obes Res* 2000, **8**:249-254.

92. Cho HJ, Park J, Lee HW, Lee YS, Kim JB: **Regulation of adipocyte differentiation and insulin action with rapamycin**. *Biochem Biophys Res Commun* 2004, **321**:942-948.

93. El-Chaar D, Gagnon A, Sorisky A: **Inhibition of insulin signaling and adipogenesis by rapamycin: effect on phosphorylation of p70 S6 kinase vs eIF4E-BP1**. *Int J Obes Relat Metab Disord* 2004, **28**:191-198.

94. Gagnon A, Lau S, Sorisky A: **Rapamycin-sensitive phase of 3T3-L1 preadipocyte differentiation after clonal expansion**. *J Cell Physiol* 2001, **189**:14-22.

95. Kim JE, Chen J: **Regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis**. *Diabetes* 2004, **53**:2748-2756.

96. Zhang HH, Huang J, Duvel K, Boback B, Wu S, Squillace RM, Wu CL, Manning BD: **Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway**. *PLoS ONE* 2009, **4**: e6189.

97. Le Bacquer O, Petroulakis E, Paglialunga S, Poulin F, Richard D, Cianflone K, Sonenberg N: **Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2**. *J Clin Invest* 2007, **117**:387-396.

98. Carnevalli LS, Masuda K, Frigerio F, Le Bacquer O, Um SH, Gandin V, Topisirovic I, Sonenberg N, Thomas G, Kozma SC: **S6K1 plays a critical role in early adipocyte differentiation**. *Dev Cell* 2010, **18**:763-774.

99. Kumar A, Lawrence JC Jr, Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA, Harris TE: **Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism**. *Diabetes* 2010, **59**:1397-1406.

100. Cybulski N, Polak P, Auwerx J, Rüegg MA, Hall MN: **mTOR complex 2 in adipose tissue negatively controls whole-body growth**. *Proc Natl Acad Sci U S A* 2009, **106**:9902-9907.

101. Dazert E, Hall MN: **mTOR signaling in disease**. *Curr Opin Cell Biol* 2011, **23**:744-755.

102. Yecies JL, Manning BD: **mTOR links oncogenic signaling to tumor cell metabolism**. *J Mol Med (Berl)* 2011, **89**:221-228.

103. Jalving M, Gietema JA, Lefrandt JD, de Jong S, Reyners AK, Gans RO, de Vries EG: **Metformin: taking away the candy for cancer?** *Eur J Cancer* 2010, **46**:2369-2380.

104. El-Serag HB, Rudolph KL: **Hepatocellular carcinoma: epidemiology and molecular carcinogenesis**. *Gastroenterology* 2007, **132**:2557-2576.

105. Caldwell SH, Crespo DM, Kang HS, Al-Osaimi AM: **Obesity and hepatocellular carcinoma**. *Gastroenterology* 2004, **127**:S97-S103.

106. Ho VW, Leung K, Hsu A, Luk B, Lai J, Shen SY, Minchinton AI, Waterhouse D, Bally MB, Lin W *et al.*: **A low carbohydrate, high protein diet slows tumor growth and prevents cancer initiation**. *Cancer Res* 2011, **71**:4484-4493.

107. Kalaany NY, Sabatini DM: **Tumours with PI3K activation are resistant to dietary restriction**. *Nature* 2009, **458**:725-731.

108. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, Osterreicher CH, Takahashi H, Karin M: **Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression**. *Cell* 2010, **140**:197-208.

109. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD: **Metformin and reduced risk of cancer in diabetic patients**. *BMJ* 2005, **330**:1304-1305.

110. Benjamin D, Colombi M, Moroni C, Hall MN: **Rapamycin passes the torch: a new generation of mTOR inhibitors**. *Nat Rev Drug Discov* 2011, **10**:868-880.

111. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, Tanti JF, Giorgetti-Peraldi S, Bost F: **Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1**. *Cancer Res* 2011, **71**:4366-4372.

112. Brown KA, Hunger NI, Docanto M, Simpson ER: **Metformin inhibits aromatase expression in human breast adipose stromal cells via stimulation of AMP-activated protein kinase**. *Breast Cancer Res Treat* 2010, **123**:591-596.

113. Kalender A, Selvaraj A, Kim SY, Gulati P, Brûlé S, Viollet B, Kemp BE, Bardeesy N, Dennis P, Schlager JJ *et al.*: **Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner**. *Cell Metab* 2010, **11**:390-401.

114. Larsson O, Morita M, Topisirovic I, Alain T, Blouin MJ, Pollak M, Sonenberg N: **Distinct perturbation of the translatome by the antidiabetic drug metformin**. *Proc Natl Acad Sci U S A* 2012, **109**:8977-8982.

115. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, Sabatini DM: **A unifying model for mTORC1-mediated regulation of mRNA translation**. *Nature* 2012, **485**:109-113.

116. Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, Shi EY, Stumpf CR, Christensen C, Bonham MJ *et al.*: **The translational landscape of mTOR signalling steers cancer initiation and metastasis**. *Nature* 2012, **485**:55-61.

117. Guertin DA, Sabatini DM: **The pharmacology of mTOR inhibition**. *Sci Signal* 2009, **2**:pe24.

118. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray NS: **An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1**. *J Biol Chem* 2009, **284**:8023-8032.

119. Dowling RJ, Topisirovic I, Alain T, Bidinosti M, Fonseca BD, Petroulakis E, Wang X, Larsson O, Selvaraj A, Liu Y *et al.*: **mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs**. *Science* 2010, **328**:1172-1176.

120 • Menon S, Yecies JL, Zhang HH, Howell JJ, Nicholatos J, Harputlugil E, Bronson RT, Kwiatkowski DJ, Manning BD: **Chronic activation of mTOR complex 1 is sufficient to cause hepatocellular carcinoma in mice**. *Sci Signal* 2012, **5**:ra24.
This is the first study that shows that hyperactivation of mTORC1 signaling in the liver results in cancer

121. Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J, Mizuno K, Hasegawa G, Kishimoto H, Iizuka M *et al.*: **Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas**. *J Clin Invest* 2004, **113**:1774-1783.

122. Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, Sheen JH, Mullholland DJ, Magnuson MA, Wu H, Sabatini DM: **mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice**. *Cancer Cell* 2009, **15**:148-159.

123. Perez-Gomez C, Campos-Sandoval JA, Alonso FJ, Segura JA, Manzanares E, Ruiz-Sanchez P, Gonzalez ME, Marquez J, Mates JM: **Co-expression of glutaminase K and L isoenzymes in human tumour cells**. *Biochem J* 2005, **386**:535-542.

124. Tennant DA, Duran RV, Boulahbel H, Gottlieb E: **Metabolic transformation in cancer**. *Carcinogenesis* 2009, **30**:1269-1280.

125. Seltzer MJ, Bennett BD, Joshi AD, Gao P, Thomas AG, Ferraris DV, Tsukamoto T, Rojas CJ, Slusher BS, Rabinowitz JD *et al.*: **Inhibition of glutaminase preferentially slows growth of glioma cells with mutant IDH1**. *Cancer Res* 2010, **70**:8981-8987.

126. Wang JB, Erickson JW, Fuji R, Ramachandran S, Gao P, Dinavahi R, Wilson KF, Ambrosio AL, Dias SM, Dang CV *et al.*: **Targeting mitochondrial glutaminase activity inhibits oncogenic transformation**. *Cancer Cell* 2010, **18**:207-219.

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Cell Biology

mTOR signaling in cellular and organismal energetics

Verena Albert and Michael N Hall

Mammalian TOR (mTOR) signaling controls growth, metabolism and energy homeostasis in a cell autonomous manner. Recent findings indicate that mTOR signaling in one tissue can also affect other organs thereby affecting whole body metabolism and energy homeostasis in a non-cell autonomous manner. It is thus not surprising that mTOR signaling mediates aging and is often deregulated in metabolic disorders, such as obesity, diabetes and cancer. This review discusses the regulation of cellular and whole body energy metabolism by mTOR, with particular focus on the non-cell autonomous function of mTOR.

Addresses
Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Corresponding author: Hall, Michael N (m.hall@unibas.ch)

Current Opinion in Cell Biology 2015, **33**:55–66

This review comes from a themed issue on **Cell regulation**

Edited by **Johan Auwerx** and **Jodi Nunnari**

http://dx.doi.org/10.1016/j.ceb.2014.12.001

Introduction

The highly conserved Target of Rapamycin (TOR) signaling pathway is a central regulator of growth and metabolism in all eukaryotes [1–5]. The serine/threonine protein kinase TOR forms two structurally and functionally distinct complexes, termed TOR Complex 1 (TORC1) and TORC2. Mammalian TORC1 (mTORC1) contains mTOR, mammalian lethal with sec-13 protein 8 (mLST8), and regulatory associated protein of mammalian target of rapamycin (raptor). mTORC1 is activated by nutrients, growth factors, and cellular energy, and is inhibited by the macrolide rapamycin [5]. Well characterized down-stream targets of mTORC1 are ribosomal protein S6 kinase (S6K), eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs), and the autophagy activating kinase ULK1 [6]. mTORC1 positively regulates anabolic processes, such as protein synthesis, ribosome biogenesis, transcription, lipid synthesis, nucleotide biosynthesis, and nutrient uptake, while inhibiting catabolic processes such as autophagy [4,5]. mTORC2 contains mTOR, mLST8, mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and rapamycin-insensitive companion of mTOR (rictor), and is activated by growth factors via association with ribosomes [7]. mTORC2 signaling regulates many cellular processes via the AGC kinase family members Akt, serum/glucocorticoid regulated kinase (SGK), and protein kinase C (PKC) [8,9].

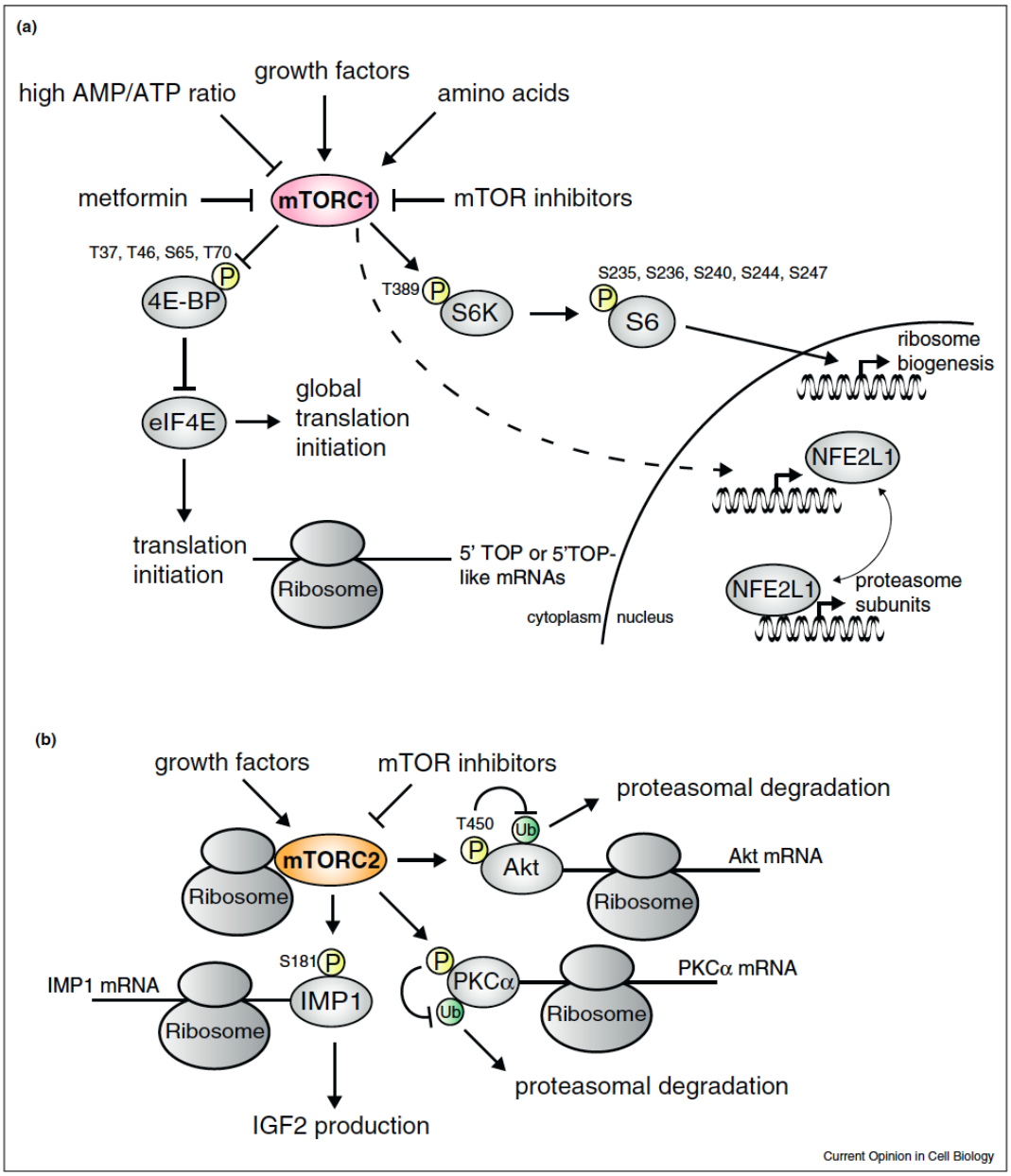
Considering that mTOR signaling is regulated by nutrients, growth factors and cellular energy status, and in turn stimulates anabolic processes, it can be viewed as a central signaling node that adapts metabolism to environmental conditions [5,10]. Aberrant energy homeostasis is a common denominator in metabolic disorders such as obesity, diabetes, and cancer. Hence, it is not surprising that deregulated mTOR signaling is often associated with these diseases. Here we review the major recent findings on the regulation of cellular energy metabolism by mTOR signaling with particular focus on protein synthesis, mitochondrial metabolism and nucleotide biosynthesis. Furthermore, we discuss how this regulation impacts cellular and organismal energetics and leads to the development of metabolic disorders.

mTOR and cellular energetics

mTOR and protein synthesis

Protein synthesis is one of the most energy demanding processes in the cell. It is therefore essential that protein synthesis occurs only under energetically favorable conditions. 4E-BPs and S6K are the main regulators of protein synthesis downstream of mTORC1 [6,11] (Figure 1a). 4E-BPs are translational repressors which upon phosphorylation by mTORC1 dissociate from eIF4E. This leads to binding of eIF4G to eIF4E at the 5′-end of mRNAs and promotes cap-dependent translation initiation. S6K indirectly affects protein synthesis by phosphorylating S6, leading to the expression of mRNAs involved in ribosome biogenesis [12•]. Whole body S6K1 and S6K2 double knockout mice or mice expressing a phospho-deficient version of S6 display a strong reduction in expression of ribosome biogenesis genes [12•,13]. S6K also phosphorylates a variety of other proteins directly or indirectly involved in the regulation of translation or mRNA processing, such as eIF4B, eukaryotic elongation factor 2 kinase (eEF2K), programmed cell death 4 (PDCD4), nuclear cap-binding protein subunit 1 (CBP80), and S6K1 Aly/REF-like substrate (SKAR) [14–20]. However, experiments using S6K-deficient liver or muscle cells failed to reveal an effect on global protein synthesis [12•,21]. Consistent with this latter observation, treatment of cells with the mTORC1 specific inhibitor rapamycin, which effectively prevents S6K activation, results in only a slight decrease in the

Figure 1



Mammalian target of rapamycin (mTOR) controls protein synthesis. **(a)** mTOR complex 1 (mTORC1) phosphorylates eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP) at multiple sites to initiate translation. Phosphorylation of 4E-BP by mTORC1 leads to 4E-BP inactivation and thus release of eIF4E. Once released, eIF4E induces cap-dependent translation initiation of mRNAs including mRNAs containing 5′TOP or 5′TOP-like sequences. mTORC1 activates ribosomal S6 kinase (S6K) through phosphorylation of T389. Active S6K phosphorylates S6 at multiple sites to stimulate ribosome biogenesis via transcription of ribosome subunits. mTORC1 stimulates protein degradation by activating transcription of nuclear factor erythroid-derived 2-related factor 1 (NFE2L1) that acts as a transcription factor for proteasome subunit genes. **(b)** Upon growth

overall translation rate [22••,23••,24]. Interestingly, a recent study by Faller *et al.* [25] demonstrated in a mouse model for colorectal cancer that mTORC1 stimulates translation elongation and thereby global protein synthesis via S6K-dependent phosphorylation of eEF2K. This finding suggests that mTORC1-S6K might indeed affect global translation, and in particular translation elongation, but under specific pathological conditions. However, in general, the mTORC1-S6K axis seems to affect translation mainly at the level of ribosome biogenesis. Complete inhibition of mTOR signaling using ATP-competitive inhibitors, such as Torin1, INK128, or PP242, strongly reduces global mRNA translation [22••,23••]. The role of mTOR in global translation seems to be via mTORC1-mediated inhibition of 4E-BP since Torin1 treatment has only minor effects on translation in 4E-BP-deficient cells [23••].

Besides its effect on global translation, four recent studies demonstrate that mTORC1 strongly affects translation of specific subsets of mRNAs [22••,23••,26••,27••]. Surprisingly, the identity of these mRNAs varies substantially between the different studies. Two of the studies found that mTORC1 specifically regulates translation of mRNAs containing 5′-terminal oligopyrimidine tract (5′TOP, cytosine at the cap site, followed by a stretch of 4–15 pyrimidines) or 5′TOP-like sequences [22••,23••] (Figure 1a). Interestingly, these mRNAs encode proteins belonging to the translation machinery, such as ribosomal proteins or elongation factors. Additionally, Thoreen *et al.* [23••] also identified a significant number of 5′TOP or 5′TOP-like mRNAs encoding proteins involved in cellular invasion and metastasis. The third study found that mTORC1 signaling affects mainly mRNAs encoding mitochondrial proteins and proteins involved in cell cycle progression [27••]. Since mTORC1 seems to preferentially regulate 5′TOP or 5′TOP-like mRNAs, it is tempting to assume that the mRNAs encoding mitochondrial proteins and cell cycle progression factors also contain 5′TOP or 5′TOP-like sequences. The above findings suggest that activation of mTORC1 signaling specifically induces translation of mRNAs encoding the translation machinery, proteins involved in cell cycle progression, mitochondrial proteins, and metastasis factors to promote cancer development. Upregulating translation of some of these mRNAs results in increased mitochondrial ATP production and elevated protein synthesis, thus ensuring continuous cell growth. Hence, targeting 5′TOP and 5′TOP-like mRNAs might prove a powerful cancer therapy. Thoreen *et al.* [23••] suggests that 5′TOP or 5′TOP-like sequences mediate 4E-BP-dependent regulation of translation, although other studies suggest that regulation by 4E-BP does not correlate with these sequences (reviewed in [28]). Future studies should clarify which downstream effector(s) mediates mTORC1-dependent translation of 5′TOP mRNAs. Interestingly, a recent study by Zhang *et al.* [29•] found that mTORC1 signaling positively regulates expression of genes encoding proteasome subunits in a nuclear factor erythroid-derived 2-related factor 1 (NFE2L1)-dependent manner. Thus, mTORC1 signaling appears to coordinate protein levels by regulating both protein synthesis and degradation.

The role of mTORC2 in protein synthesis is not clear. It has been shown that upon growth factor stimulation, mTORC2 is activated through association with the ribosome [7]. This is interesting because ribosomal content reflects the growth capacity of a cell. By making mTORC2 activation dependent on ribosomal content, the cell ensures that mTORC2 signaling is activated only under favorable growth conditions. Intriguingly, ribosomes vastly outnumber mTORC2, suggesting that the mTORC2-ribosome interaction is tightly regulated or that mTORC2 is regulated by a specific subset of ribosomes [30••]. Cancer cells with hyperactive PI3K signaling display increased mTORC2-ribosome association and thus enhanced mTORC2 activity [7]. Moreover, a ribosomal protein deficiency inhibits Akt-driven tumorigenesis [31], indicating that mTORC2-ribosome association plays a role in cancer development. mTORC2-ribosome association is independent of translation. Increased mTORC2-ribosome association upon growth factor stimulation and subsequent mTORC2 activation occur even in the absence of translation. Hence, translation does not seem to modulate mTORC2 signaling. However, it has been shown that mTORC2 specifically associates with ribosomes translating mTORC2 substrates [32,33] (Figure 1b). mTORC2 co-translationally phosphorylates its nascent targets. For example, co-translational phosphorylation of Akt at T450 and PKC prevents their ubiquitylation and subsequent degradation [32]. On the other hand, co-translational phosphorylation of IGF2 mRNA binding protein 1 (IMP1) at S181 leads to increased IGF2 production due to enhanced splicing and translation of IGF2 mRNA [33]. Identification of other co-translationally phosphorylated mTORC2 substrates will provide better understanding of the role of mTORC2 in translation.

mTOR and mitochondria

Mitochondria are the main site for ATP production. ATP is generated by the electron transport chain (ETC) via transport of protons through ATP synthase into the inner mitochondrial matrix. ATP is the main energy source for

(Figure 1 Legend Continued) factor stimulation, mTORC2 associates with ribosomes where it co-translationally phosphorylates its targets. Co-translational phosphorylation of Akt at T450 and protein kinase C α (PKCα) prevents Akt and PKCα ubiquitylation and proteasomal degradation. Co-translational phosphorylation of insulin-like growth factor 2 (IGF2) mRNA binding protein (IMP1) at S181 stabilizes IGF2 mRNA, thereby inducing IGF2 production.

most biological processes, and mitochondrial function is thus essential for cell survival. In line with this, other important metabolic pathways, such as fatty acid oxidation and the tricarboxylic acid (TCA) cycle are situated in the mitochondria. Moreover, mitochondria are important regulators of apoptosis and cell death [34]. The importance of mitochondrial function in cellular homeostasis is underscored by the fact that mitochondrial metabolism is often deregulated in metabolic disorders. For example, to maintain enhanced growth rates, cancer cells shift their mitochondrial bioenergetic state from ATP production to increased de novo synthesis of cellular building blocks. This is usually achieved by compensating generation of ATP through glycolysis and a decrease in oxidative phosphorylation in order to use TCA cycle intermediates for de novo synthesis of cellular building blocks [35]. Hence, an altered metabolic state due to altered mitochondrial function has emerged as an important mediator of enhanced growth rates in cancer cells.

Since mTOR signaling stimulates ATP-consuming processes such as translation, nucleotide biosynthesis and nutrient uptake, mitochondrial function and mTOR signaling are closely intertwined. mTORC1 activity correlates with mitochondrial activity [36]. Rapamycin decreases mitochondrial metabolism in cultured cells. Hence, mTORC1 signaling seems to positively regulate mitochondrial activity (Figure 2a). In skeletal muscle, mTORC1 activity is a crucial determinant of oxidative metabolism and mitochondrial function. Inactivation of mTORC1 signaling in skeletal muscle through knockout of *raptor* or *mTOR* results in a strong decrease in mitochondrial function and oxidative metabolism [37,38]. On the other hand, hyper-activation of mTORC1 signaling in skeletal muscle through knockout of *tuberous sclerosis complex 1* (*TSC1*) leads to enhanced oxidative activity [39]. Despite this clear evidence for a positive correlation between mTORC1 signaling and mitochondrial function in skeletal muscle, the underlying molecular events are not fully understood. mTORC1 hyper-activation through knockdown of TSC2 in C2C12 myotubes results in enhanced transcription of genes involved in mitochondrial metabolism, such as peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), PGC-1β, and nuclear respiratory factor 1 (NRF1) via interaction of mTORC1 with the transcription factor yin-yang 1 [40]. In line with this, skeletal muscle-specific *raptor* or *mTOR* knockout mice display a strong decrease in the transcript levels of PGC-1α and PGC-1α target genes [37,38]. Importantly, elevating PGC-1α levels in raptor-deficient muscle restores mitochondrial function [41]. This suggests that a decrease in PGC-1α is responsible for the reduction in oxidative capacity in mTORC1 deficient muscle. Surprisingly, despite the hyperactivation of mTORC1 and enhanced oxidative capacity in muscle-specific *TSC1* knockout (TSCmKO) mice, these mice also display a strong decrease in PGC-1α transcript levels [39]. The enhanced oxidative capacity in the muscle of TSCmKO mice could be accounted for by elevated levels of PGC-1β. In conclusion, mTORC1 signaling in skeletal muscle stimulates mitochondrial function and oxidative metabolism; however, further studies are required to elucidate the exact molecular mechanism of this regulation.

As mentioned above, mTORC1 signaling affects mitochondrial function via translation in addition to transcription. In breast cancer cells, mTORC1-4E-BP signaling specifically stimulates translation of genes encoding mitochondrial proteins [26••,27••] (Figure 2a). Consequently, knockdown of raptor in these cells decreases mitochondrial function and ATP production. Interestingly, phosphatase and tensin homolog (PTEN)-deficient mouse embryonic fibroblasts (MEFs), which exhibit increased mTORC1 activity, display increased translation of components of the ETC [42]. Importantly, 4E-BP levels are significantly decreased in these cells, suggesting that the regulation of mRNAs encoding mitochondrial proteins can be regulated in a 4E-BP-dependent manner in different contexts. To better understand the role of mTOR signaling in mitochondrial metabolism and its impact on cancer development, it would be of interest to investigate whether enhanced translation of mitochondrial proteins and thus enhanced mitochondrial function is a common feature of cancer cells with hyperactive mTORC1. Moreover, it would be interesting to test whether cancer cells with hyperactive mTORC1 are particularly sensitive to mitochondrial inhibition.

In contrast to the effects observed in skeletal muscle and breast cancer cells, mTORC1 signaling in adipose tissue seems to exert negative effects on mitochondrial activity and oxidative metabolism. Adipose tissue-specific *raptor* knockout mice display enhanced respiration in white adipose tissue (WAT) and no defect in mitochondrial gene transcription [43]. Moreover, adipose tissue-specific *Growth factor receptor-bound protein 10* (*Grb10*) knockout mice, which exhibit enhanced mTORC1 activity, display reduced PGC-1α transcript levels and concurrently decreased respiration and β-oxidation [44•]. Taken together, with the exception of adipose tissue, mTORC1 seems to stimulate mitochondrial metabolism and function. This would allow cells with high mTORC1 activity to sustain elevated growth rates by stimulating mitochondrial biogenesis and metabolism, thereby generating a feed-forward loop that increases the availability of ATP needed for anabolic processes and growth. Further studies are required to understand why mTORC1 exerts a negative influence on mitochondrial processes in adipose tissue. An explanation for the negative action of mTORC1 in adipose tissue might be that this favors energy storage in times of nutrient abundance and energy mobilization in times of famine.

The connection between mTORC2 and mitochondria is less studied. However, there are several lines of evidence suggesting that mTORC2 signaling negatively affects mitochondrial function (Figure 2b). For example, knockout of *rictor* in the liver or in brown adipose tissue (BAT) and muscle precursors results in increased expression of PGC-1α mRNA and an enhanced oxidative phenotype [45–47]. In line with this, knockdown of rictor in cells leads to enhanced oxygen consumption [36]. Moreover, breast cancer cells or MEFs deficient for mTORC2 signaling exhibit enhanced ATP production without any effect on mitochondrial number [27••,30••]. The molecular events accounting for this finding are just starting to become clear. Betz *et al.* reported that mTORC2 localizes to mitochondria-associated membranes (MAM) — a subcompartment of the endoplasmic reticulum (ER) — in response to growth factor stimulation [30••,48]. Importantly, localization of mTORC2 to MAM seems to directly affect mitochondrial function (Figure 2b). For example, mTORC2 signaling decreases ATP levels and mitochondrial activity via an Akt-dependent recruitment of Hexokinase 2 (HK2) to MAM. This results in stimulation of glycolysis and a subsequent decrease in mitochondrial inner membrane potential and hence reduced mitochondrial activity. Furthermore, mTORC2 signaling at MAM inhibits calcium release from the ER via phosphorylation of the inositol triphosphate receptor (IP3R) in an Akt-dependent fashion, thereby regulating apoptosis. Interestingly, liver-specific *rictor* knockout (LiRiKO) mice display very similar metabolic alterations compared to mice with a liver-specific disruption of MAM, including increased gluconeogenesis, hyperinsulinemia and glucose intolerance [49]. Hence, mTORC2 signaling seems to affect growth and metabolism largely via regulation of mitochondria-regulated processes at MAM. The connection between mTORC2 and mitochondria also seems to be relevant for cancer. A recent genetic screen revealed that cancer cells with high mTORC2 signaling display increased dependence on mitochondrial function and in particular HK2 [50]. Targeting mTORC2-regulated mitochondrial functions might provide promising therapeutic targets against cancer and metabolic disorders.

mTOR and nucleotide biosynthesis

De novo nucleotide biosynthesis provides building blocks for both DNA and RNA and is thus an important determinant of a cell's growth capacity. Increasing evidence suggests that mTORC1 signaling directly promotes de novo nucleotide biosynthesis. mTORC1 stimulates transcription of pentose phosphate pathway (PPP) genes at least in part via the transcription factor sterol regulatory element-binding protein (SREBP) [51]. This increases the intracellular levels of ribose that in turn can be used for de novo nucleotide biosynthesis. Additionally, two recent studies demonstrated that mTORC1 stimulates de novo pyrimidine biosynthesis through S6K1-mediated phosphorylation and activation of the CAD protein. CAD encodes the three distinct enzymatic activities *c*arbamoyl phosphate synthetase 2, *a*spartate transcarbamoylase, and *d*ihydroorotase, and thereby mediates the three initial and rate limiting steps in de novo pyrimidine biosynthesis [52•,53•]. Thus, mTORC1 increases synthesis of ribose and pyrimidines to promote DNA and RNA synthesis. Pyrimidines are also high-energy molecules that drive specific cellular reactions. For example, UTP and CTP activate carbohydrates for transfer to other molecules and CTP is an energy source for lipid synthesis. So far, there is no evidence that mTORC2 is involved in nucleotide biosynthesis. However, mTORC2 signaling is known to activate SREBPs [45] and might thereby stimulate expression of PPP genes.

mTOR and organismal energetics

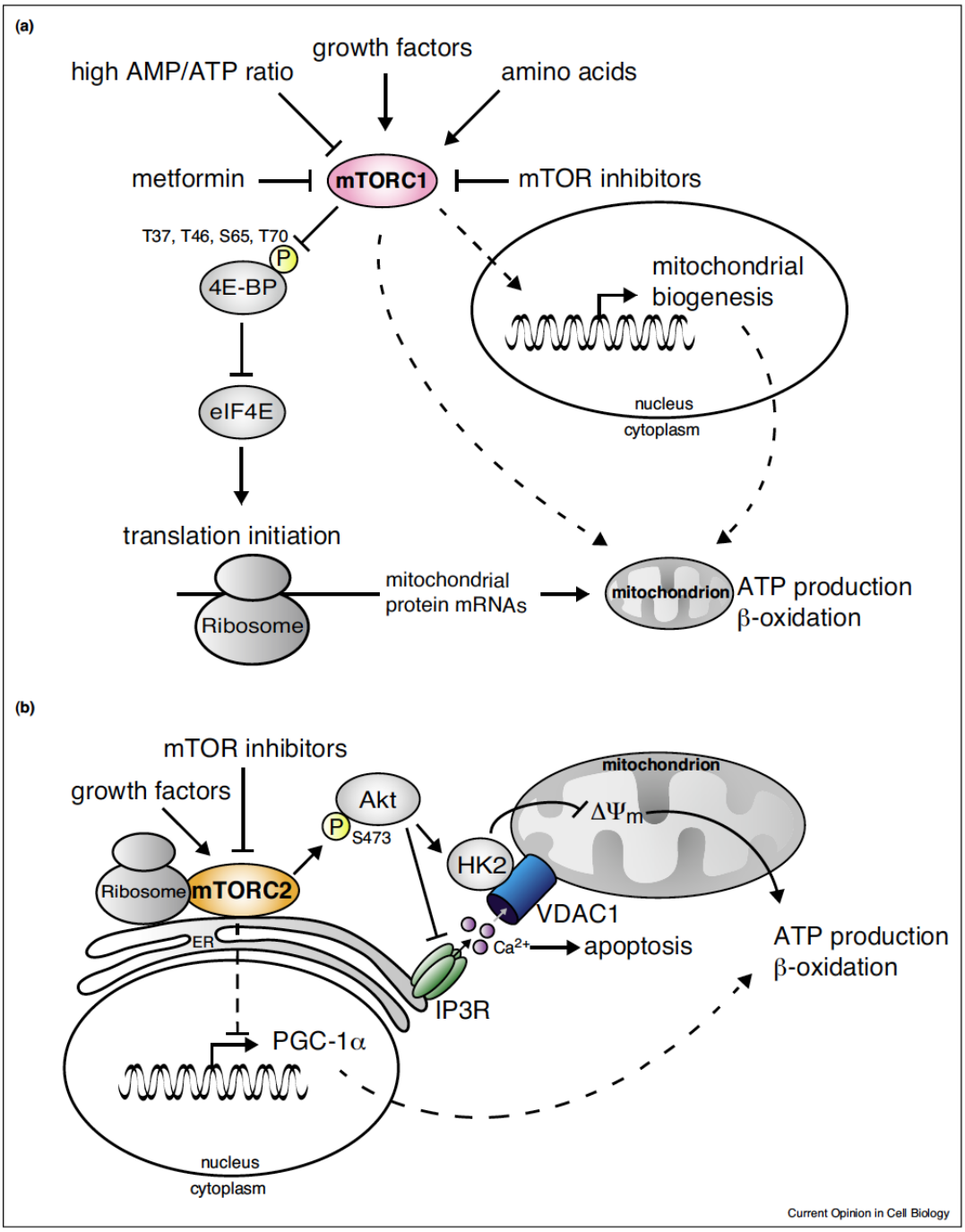
A large body of work over many years has led to an understanding of the molecular mechanisms by which mTOR regulates cellular metabolism and growth. However, to fully grasp the impact of mTOR signaling on growth and metabolism, mTOR signaling must also be studied in the context of the entire organism. Studies on mice with tissue or cell type-specific deletions of different mTOR signaling components have revealed important effects of tissue-specific mTOR signaling on other organs, thereby affecting whole body physiology. In this section we discuss the whole body effects of modulating mTOR signaling in a specific tissue (Table 1).

mTOR in adipose tissue

Adipose tissue is important for nutrient homeostasis and energy balance. It serves as an energy depot and is an important endocrine organ that secretes hormones and adipokines to regulate whole body metabolism [54]. Because of the central role of mTOR signaling in nutrient sensing and metabolism, altered mTOR activity in adipose tissue has profound effects on whole body metabolism.

Adipose tissue-specific *raptor* knockout mice with impaired mTORC1 signaling in adipose tissue display increased energy expenditure due to mitochondrial uncoupling in WAT. This results in lean mice and protection against diet-induced obesity [43]. Moreover, these mice display improved glucose homeostasis on a high fat diet (HFD), due to increased insulin signaling in skeletal muscle. Conversely, mice with enhanced mTORC1 signaling in adipose tissue due to *Grb10* knockout show the opposite phenotype [44•]. These mice display decreased energy expenditure that results in obesity and decreased systemic insulin sensitivity. Moreover, enhanced mTORC1 signaling in adipose tissue impairs thermogenesis, due to decreased mitochondrial uncoupling in both BAT and WAT. Taken together, mTORC1 signaling in adipose tissue negatively affects whole body energy expenditure, systemic insulin sensitivity and thermogenesis (Table 1). Thus, mTORC1 signaling activity in adipose tissue has fundamental

Figure 2


(a)
growth factors
high AMP/ATP ratio
amino acids
metformin
mTORC1
mTOR inhibitors
T37, T46, S65, T70
P
4E-BP
eIF4E
mitochondrial
biogenesis
nucleus
cytoplasm
translation initiation
Ribosome
mitochondrial
protein mRNAs
mitochondrion
ATP production
β-oxidation
(b)
mTOR inhibitors
growth factors
Akt
P
S473
mitochondrion
ΔΨm
HK2
Ribosome
mTORC2
VDAC1
ER
Ca2+
apoptosis
ATP production
β-oxidation
IP3R
PGC-1α
nucleus
cytoplasm
Current Opinion in Cell Biology

impact on whole body metabolism and energy balance. Interestingly, mTORC1 signaling is enhanced in WAT of HFD fed mice as compared to mice fed a standard diet [43]. Hyper-activation of mTORC1 signaling in adipose tissue upon HFD feeding might play a role in the development of metabolic complications during obesity. Decreasing mTORC1 signaling specifically in adipose tissue could thus be a powerful strategy to counter diet-induced metabolic disorders.

Inactivation of mTORC2 signaling in adipose tissue also profoundly affects whole body metabolism. HFD fed mice with adipose tissue-specific deletion of *rictor* (AdRiKO mice) are characterized by increased body size due to enhanced growth of non-adipose tissues [55,56]. This increase in animal size is explained by increased levels of circulating IGF-1 and insulin. The increase in circulating insulin in AdRiKO mice is due to a disproportionately enlarged pancreas, whereas the enhanced IGF-1 levels are derived from the liver. The increase in circulating insulin also accounts for an increase in glucose tolerance upon HFD despite slight insulin resistance in muscle and liver. Hence mTORC2 signaling in adipose tissue is a crucial regulator of both liver and pancreas metabolism through which it affects whole body growth and systemic glucose and insulin homeostasis (Table 1).

A recent study investigated the role of mTORC2 signaling in Myf5-positive precursors of BAT and skeletal muscle [47]. This cell-type specific ablation of *rictor* leads to a shift in BAT toward a more oxidative metabolism associated with increased mitochondrial uncoupling. This enhances whole body energy expenditure, protecting the mice against diet-induced obesity. Hence, in contrast to inactivation of mTORC2 signaling in mature white and brown adipocytes (AdRiKO mice), absence of mTORC2 signaling specifically in Myf5 positive precursor cells enhances oxidative metabolism thereby increasing metabolic rate without aberrant effects on body size (Table 1).

mTOR in liver

The liver plays a key role in lipid metabolism, and the involvement of both mTORC1 and mTORC2 in this process has already been extensively reviewed elsewhere [57–59]. We therefore focus below on other mTOR-regulated metabolic processes in the liver and their impact on whole body metabolism. mTORC1 signaling in the liver affects systemic glucose and insulin homeostasis. In the liver of liver-specific *TSC1* knockout (L-*TSC1* KO) mice, chronic activation of mTORC1 signaling exerts strong inhibitory feedback on IRS-1, resulting in decreased Akt signaling. This impairs hepatic glycolytic flux and hence glucose clearance from the blood, leading to glucose intolerance [60–62]. Conversely, liver-specific *raptor* knockout mice display increased systemic glucose tolerance that can be explained by enhanced Akt signaling and insulin-induced glucose uptake in the liver [63,64]. Hence, hepatic mTORC1 signaling results in systemic regulation of glucose and insulin signaling, most probably due to its effect on Akt and hepatic glucose uptake (Table 1).

A recent study by Cornu *et al.* [65••] demonstrated another interesting connection between hepatic mTORC1 signaling and whole body physiology in L-*TSC1* KO mice. Hyper-activation of mTORC1 signaling upon fasting causes metabolic stress due to systemic and hepatic glutamine depletion and thereby an inability of glutaminolysis to sustain the TCA cycle. By an unknown mechanism, this metabolic stress stimulates PGC-1α-dependent FGF-21 production in the liver. FGF-21 in turn acts on the central nervous system to decrease locomotor activity and body temperature [66] (Table 1). The glutamine depletion observed in starved L-*TSC1* KO mice is consistent with the finding that mTORC1 inhibition increases intracellular glutamine levels [67•]. Moreover, glucose-limited TSC-deficient cells are addicted to glutamine as an alternative carbon source, further accentuating the role of mTORC1 in regulating glutamine metabolism [68,69]. Interestingly, many tumors are glutamine addicted, leading to systemic glutamine depletion in cancer patients [70–72]. Cornu *et al.* demonstrated that mTORC1 activity correlates with FGF-21 expression in human liver tumors. This suggests a link between metabolic stress-induced FGF-21 expression, glutamine addiction, and hyperactive mTORC1 signaling in tumors. Thus, treatment with mTOR inhibitors might have beneficial effects at the tumor and whole body level in patients with glutamine-addicted cancers.

Similar to hepatic mTORC1, mTORC2 signaling in the liver also affects systemic glucose and insulin

(Figure 2 Legend) Mammalian target of rapamycin (mTOR) affects mitochondria. **(a)** mTOR complex 1 (mTORC1) promotes translation of mRNAs encoding mitochondrial proteins, through inhibition of eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP). mTORC1 stimulates mitochondrial ATP production and β-oxidation by inducing mitochondrial biogenesis and possibly by other mechanisms. **(b)** mTORC2 inhibits transcription of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), thereby negatively affecting mitochondrial activity. Upon growth factor stimulation, mTORC2 associates with mitochondria associated membranes (MAM) of the endoplasmic reticulum (ER) to affect mitochondrial function via activation of Akt by phosphorylating it at S473. Akt phosphorylates and inactivates inositol triphosphate receptor (IP3R), which leads to inhibition of calcium release from the ER, thereby blocking apoptosis. Akt also phosphorylates and activates Hexokinase 2 (HK2). Active HK2 decreases the inner mitochondrial membrane potential (ΔΨm), thereby inhibiting mitochondrial activity. VDAC1, voltage dependent anion channel 1.

Table 1

Effects of altered mTOR signaling in different organs on whole body metabolism. Details are discussed in the text

Organ with altered mTOR signaling	Effects on other organs or systemic metabolism	Mouse models
Adipose tissue		
mTORC1 inactivation	Energy expenditure ↑ Skeletal muscle insulin sensitivity ↑ Circulating leptin ↓ Locomotor activity ↓ Diet-induced obesity ↓ Glucose tolerance on HFD ↑	Adipose tissue-specific *raptor* KO mice [43]
mTORC1 hyper-activation	Energy expenditure ↓ Thermogenesis ↓ Systemic insulin sensitivity ↓ Circulating insulin on HFD ↑ Glucose tolerance ↓	Adipose tissue-specific *Grb10* KO mice [44•]
mTORC2 inactivation	Body growth ↑ Systemic insulin sensitivity ↓ Glucose tolerance ↑ Circulating insulin ↑ Hepatic steatosis ↑	Adipose tissue-specific *rictor* KO mice [55,56]
Brown adipose tissue/muscle progenitors		
mTORC2 inactivation	Energy expenditure ↑ Thermogenesis ↑ Hepatic steatosis ↓ Diet-induced obesity ↓ Glucose tolerance on HFD ↑	Myf5 positive BAT/muscle precursor cell-specific *rictor* KO mice [47]
Liver/hepatocytes		
mTORC1 inactivation	Circulating cholesterol ↓ Glucose tolerance ↑	Liver-specific *raptor* KO mice [63,64]
mTORC1 hyper-activation	Locomotor activity ↓ Body temperature ↓ Systemic glutamine levels ↓ Glucose tolerance ↓ Circulating ketone bodies ↓	Liver-specific *TSC1* KO mice [60,61,62,65••]
mTORC2 inactivation	Systemic insulin sensitivity ↓ Circulating insulin ↑ Glucose tolerance ↓ Circulating triglycerides ↓ Circulating cholesterol ↓ Lifespan (males only) ↓	Liver-specific *rictor* KO mice [45,46,73,74•]
Skeletal muscle		
mTORC1 inactivation	Adipose tissue mass ↓ Diet-induced obesity ↓ Glucose tolerance ↓ Lifespan ↓	Skeletal muscle-specific *raptor* KO mice [37]
mTORC1 hyper-activation	Adipose tissue mass ↓	Skeletal muscle-specific *TSC1* KO mice [39,82]
mTORC2 inactivation	Glucose tolerance ↓	Skeletal muscle-specific *rictor* KO mice [37,83]
Hypothalamus (POMC neurons)		
mTORC1 hyper-activation	Adipose tissue mass ↑ Circulating leptin ↑ Food intake ↑	POMC neuron-specific *TSC1* KO mice [89]
mTORC2 inactivation	Adipose tissue mass ↑ Glucose tolerance ↓ Food intake ↑	POMC neuron-specific *rictor* KO mice [88•]
Hypothalamus (Agrp neurons)		
mTORC2 inactivation	Glucose tolerance ↓	Agrp-specific *rictor* KO mice [88•]

homeostasis. Liver-specific *rictor* knockout (LiRiKO) mice display increased circulating insulin levels and impaired glucose tolerance and insulin sensitivity [45,46,73,74•]. Similar to L-*TSC1*-KO mice, hepatic Akt signaling is impaired in LiRiKO mice, which results in decreased hepatic glucose uptake. However, despite the hyperinsulinemic phenotype, other metabolic organs do not display insulin resistance. Hence, hepatic

mTORC2 affects systemic glucose and insulin homeostasis mainly by affecting hepatic glucose uptake via Akt signaling [45], which could explain the similar phenotypes of L-*TSC1* KO mice and LiRiKO mice with regard to glucose homeostasis (Table 1). Interestingly, a recent study by Lamming *et al.* [74•] demonstrated that male but not female LiRiKO mice have a reduced lifespan. Since both male and female LiRiKO mice display a diabetic phenotype, the authors concluded that the decreased lifespan in male LiRiKO mice is not due to impaired glucose homeostasis. Further studies are required to elucidate the molecular mechanism via which hepatic mTORC2 regulates lifespan.

mTOR in skeletal muscle

Skeletal muscle is the largest organ in the body and modulation of its metabolic state has a substantial impact on systemic energy homeostasis [75]. Skeletal muscle furthermore accounts for the majority of insulin-stimulated glucose uptake, which is dependent on skeletal muscle Akt signaling [76,77]. Hence, impaired glucose uptake in skeletal muscle plays a key role in the development of insulin resistance. Since mTOR signaling regulates glucose homeostasis as well as other important metabolic processes, modulation of mTOR signaling in skeletal muscle has major impact on whole body energy homeostasis.

Skeletal muscle-specific *raptor* knockout (RAmKO) mice display progressive loss of both muscle and adipose tissue mass [37]. Interestingly, mice with skeletal muscle-specific over-expression of Akt are also lean and resistant to diet-induced obesity [78]. Importantly, RAmKO mice display hyperactive Akt signaling due to absence of the negative feedback loop from S6K to IRS. Hence, the loss of adipose tissue mass in RAmKO mice could be due to enhanced Akt signaling in skeletal muscle. Why does activation of Akt signaling in muscle lead to loss of adipose tissue mass? Hyperactivation of Akt signaling in RAmKO mice is associated with increased glycogenesis, as revealed by accumulation of glycogen in muscle (Table 1) [37]. The authors speculated that muscle of RAmKO mice takes up increased amounts of glucose, and thereby acts as a glucose sink. Consequently, other tissues must switch to alternative energy sources, such as fatty acids, leading to progressive loss of adipose tissue.

Because of hyperactive Akt signaling and increased glycogen stores in skeletal muscle, RAmKO mice should be more glucose tolerant. Surprisingly, despite enhanced Akt signaling in skeletal muscle, RAmKO mice are glucose intolerant [37]. This suggests that absence of mTORC1 signaling in skeletal muscle exerts a negative effect on whole body glucose homeostasis despite increased Akt signaling in skeletal muscle. Interestingly, inactivation of mTORC1 signaling in adipose tissue improves glucose tolerance [43]. This again indicates (see above) that mTORC1 in adipose tissue and skeletal muscle exert opposite effects, in this case on whole body glucose homeostasis (Table 1). It is well documented that chronic rapamycin treatment impairs glucose homeostasis in mice [73], rats [79,80], and humans [81]. Lamming *et al.* [73] suggested that the rapamycin-induced diabetic phenotype is due to inhibition of hepatic mTORC2, because rapamycin treatment does not worsen the diabetic phenotype of LiRiKO mice [45]. However, based on the phenotype of RAmKO mice [37], the negative effect of mTORC1 inactivation in skeletal muscle on glucose homeostasis could also contribute to the diabetic phenotype observed upon rapamycin treatment.

Considering that mTORC1 regulates protein synthesis, it would be expected that muscle-specific *TSC1* knockout (TSCmKO) mice display a hypertrophic phenotype. Surprisingly, and similar to RAmKO mice, TSCmKO mice are characterized by a progressive loss of both muscle and adipose tissue mass (Table 1) [82]. The progressive loss of muscle mass in TSCmKO mice is due to impaired autophagy resulting in muscle damage. Hence, too much or too little mTORC1 activity skeletal muscle can have detrimental effects on muscle and whole body homeostasis. It would be of interest to study other metabolic processes, such as glucose homeostasis, in TSCmKO mice to see whether these parameters are also regulated in a similar fashion as in RAmKO mice despite opposite effects on mTORC1 signaling in skeletal muscle.

The effects of skeletal muscle-specific ablation of *rictor* on whole body metabolism are relatively mild [37,83]. Confirming the role of mTORC2 signaling in regulating Akt-mediated glucose uptake, inactivation of mTORC2 in skeletal muscle results in glucose intolerance (Table 1). In summary, mTORC1 signaling in skeletal muscle exerts a more profound effect on whole body metabolism as compared to mTORC2 signaling. However, since mTORC2 signaling in skeletal muscle affects glucose uptake it might play a crucial role in diet-induced insulin resistance.

mTOR in hypothalamus

The arcuate nucleus (ARC) in the hypothalamus is a master regulator of whole body energy homeostasis. It contains two distinct neuronal populations, appetite stimulatory agouti-related protein/neuropeptide Y (Agrp/NPY) neurons that secrete the neuropeptides Agrp and NPY and appetite inhibitory pro-opiomelanocortin (POMC) neurons that secrete the neuropeptide POMC. Agrp/NPY neurons are activated to induce feeding while cessation of feeding is achieved through activation of POMC neurons [84–86]. Since mTOR signaling is sensitive to nutrients, it was hypothesized that mTOR might play a crucial role in regulating the function of Agrp/NPY and POMC neurons. Surprisingly, neither *TSC1* nor *rictor* deletion in Agrp neurons results in any alterations of

energy homeostasis or feeding behavior [87,88•]. The only phenotype that could be observed was mild glucose intolerance in Agrp-specific *rictor* knockout mice (Table 1). These findings suggest that normal mTOR signaling is not required for the basal function of Agrp neurons. Alternatively, compensatory mechanisms could have developed in these knockout models to circumvent the dependence on mTORC1 and mTORC2 signaling in Agrp neurons.

In contrast to knockouts in Agrp neurons, POMC-specific deletion of either *TSC1* or *rictor* results in aberrantly regulated feeding behavior [88•,89]. Mice with hyperactive mTORC1 signaling in POMC neurons display reduced expression of POMC, a concomitant increase in NPY levels, and hyperphagia-induced obesity [89] (Table 1). Interestingly, mTORC2 inactivation in POMC neurons results in a somewhat milder but still very similar phenotype to mTORC1 hyper-activation. POMC-specific *rictor* knockout mice are hyperphagic and obese [88•]. The effects of mTORC1 hyper-activation and mTORC2 inactivation could be due to impaired Akt signaling. Akt inhibits the transcription factor Forkhead-box containing protein O1 (FoxO1) through phosphorylation and nuclear exclusion [90]. Impaired Akt signaling results in nuclear FoxO1 that represses POMC expression by preventing STAT3 from binding to the POMC promoter [91]. Hence, mTORC2 signaling in POMC neurons is crucial to induce cessation of feeding under nutrient rich conditions, by increasing POMC expression.

Conclusions and future perspectives

This review emphasizes the importance of mTOR signaling in cellular and organismal energy homeostasis. mTOR signaling has impact on cellular energetics by regulating protein, lipid and nucleotide synthesis, mitochondrial homeostasis, and other metabolic processes. Moreover, tissue-specific modulation of mTOR signaling has profound effects on other organs and whole body energy homeostasis. Hence, the non-cell autonomous effects of mTOR signaling might substantially contribute to metabolic alterations observed in obesity, diabetes and cancer. For example, both adipose and muscle-specific *raptor* knockout mice are lean and resistant to diet-induced obesity. Additionally, aberrant activation of mTORC1 in the context of cancer, could potentially lead to metabolic stress, affecting whole body physiology and behavior. Understanding how mTOR signaling in a particular tissue affects metabolism in other organs is thus important in the development of drugs targeting mTOR signaling to treat metabolic disorders and cancer.

Acknowledgements

We acknowledge support from the Swiss National Science Foundation, the Loius-Jeantet Foundation, the European Research Council, the Werner Siemens Foundation (VA), and the Canton of Basel-Stadt.

References and recommended reading

Papers of particular interest, published within the period of review,

have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Wullschleger S, Loewith R, Hall MN: **TOR signaling in growth and metabolism**. *Cell* 2006, **124(3)**:471-484.
2. Laplante M, Sabatini DM: **mTOR signaling in growth control and disease**. *Cell* 2012, **149(2)**:274-293.
3. Loewith R, Hall MN: **Target of rapamycin (TOR) in nutrient signaling and growth control**. *Genetics* 2011, **189(4)**:1177-1201.
4. Shimobayashi M, Hall MN: **Making new contacts: the mTOR network in metabolism and signalling crosstalk**. *Nat Rev Mol Cell Biol* 2014, **15(3)**:155-162.
5. Dibble CC, Manning BD: **Signal integration by mTORC1 coordinates nutrient input with biosynthetic output**. *Nat Cell Biol* 2013, **15(6)**:555-564.
6. Ma XM, Blenis J: **Molecular mechanisms of mTOR-mediated translational control**. *Nat Rev Mol Cell Biol* 2009, **10(5)**:307-318.
7. Zinzalla V *et al.*: **Activation of mTORC2 by association with the ribosome**. *Cell* 2011, **144(5)**:757-768.
8. Cybulski N, Hall MN: **TOR complex 2: a signaling pathway of its own**. *Trends Biochem Sci* 2009, **34(12)**:620-627.
9. Sparks CA, Guertin DA: **Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy**. *Oncogene* 2010, **29(26)**:3733-3744.
10. Kim SG, Buel GR, Blenis J: **Nutrient regulation of the mTOR complex 1 signaling pathway**. *Mol Cells* 2013, **35(6)**:463-473.
11. Mamane Y *et al.*: **mTOR: translation initiation and cancer**. *Oncogene* 2006, **25(48)**:6416-6422.
12. • Chauvin C *et al.*: **Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program**. *Oncogene* 2014, **33(4)**:474-483.
This study demonstrates that S6 regulates transcription of ribosome proteins.
13. Ruvinsky I, Meyuhas O: **Ribosomal protein S6 phosphorylation: from protein synthesis to cell size**. *Trends Biochem Sci* 2006, **31(6)**:342-348.
14. Raught B *et al.*: **Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases**. *EMBO J* 2004, **23(8)**:1761-1769.
15. Shahbazian D *et al.*: **The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity**. *EMBO J* 2006, **25(12)**:2781-2791.
16. Peschiaroli A *et al.*: **SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response**. *Mol Cell* 2006, **23(3)**:319-329.
17. Wang X *et al.*: **Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase**. *EMBO J* 2001, **20(16)**:4370-4379.
18. Wilson KF, Wu WJ, Cerione RA: **Cdc42 stimulates RNA splicing via the S6 kinase and a novel S6 kinase target, the nuclear cap-binding complex**. *J Biol Chem* 2000, **275(48)**:37307-37310.
19. Richardson CJ *et al.*: **SKAR is a specific target of S6 kinase 1 in cell growth control**. *Curr Biol* 2004, **14(17)**:1540-1549.
20. Ma XM *et al.*: **SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs**. *Cell* 2008, **133(2)**:303-313.
21. Mieulet V *et al.*: **S6 kinase inactivation impairs growth and translational target phosphorylation in muscle cells maintaining proper regulation of protein turnover**. *Am J Physiol Cell Physiol* 2007, **293(2)**:C712-C722.

22. •• Hsieh AC *et al.*: **The translational landscape of mTOR signalling steers cancer initiation and metastasis**. *Nature* 2012, **485(7396)**:55-61.
This study together with [23••] demonstrates that mTORC1 regulates translation of mRNAs containing 5'TOP or 5'TOP-like sequences in a 4E-BP-dependent fashion.

23. •• Thoreen CC *et al.*: **A unifying model for mTORC1-mediated regulation of mRNA translation**. *Nature* 2012, **485(7396)**:109-113.
This study together with [22••] demonstrates that mTORC1 regulates translation of mRNAs containing 5'TOP or 5'TOP-like sequences in a 4E-BP-dependent fashion.

24. Guertin DA, Sabatini DM: **The pharmacology of mTOR inhibition**. *Sci Signal* 2009, **2(67)**:ppe24.

25. Faller WJ *et al.*: **mTORC1-mediated translational elongation limits intestinal tumour initiation and growth**. *Nature* 2014. [Epub ahead of print].

26. •• Larsson O *et al.*: **Distinct perturbation of the translatome by the antidiabetic drug metformin**. *Proc Natl Acad Sci U S A* 2012, **109(23)**:8977-8982.
This study together with [27••] demonstrates that mTORC1 regulates translation of mRNAs encoding mitochondrial proteins in a 4E-BP-dependent fashion.

27. •• Morita M *et al.*: **mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation**. *Cell Metab* 2013, **18(5)**:698-711.
This study together with [26••] demonstrates that mTORC1 regulates translation of mRNAs encoding mitochondrial proteins in a 4E-BP-dependent fashion.

28. Meyuhas O, Kahan T: **The race to decipher the top secrets of TOP mRNAs**. *Biochim Biophys Acta* 2014. [Epub ahead of print].

29. • Zhang Y *et al.*: **Coordinated regulation of protein synthesis and degradation by mTORC1**. *Nature* 2014, **513(7518)**:440-443.
This study demonstrates that mTORC1 signaling stimulates proteasomal degradation.

30. •• Betz C *et al.*: **Feature Article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology**. *Proc Natl Acad Sci U S A* 2013, **110(31)**:12526-12534.
This study demonstrates that mTORC2 associates at MAM to regulate mitochondrial metabolism.

31. Hsieh AC *et al.*: **Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E**. *Cancer Cell* 2010, **17(3)**:249-261.

32. Oh WJ *et al.*: **mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide**. *EMBO J* 2010, **29(23)**:3939-3951.

33. Dai N *et al.*: **mTOR complex 2 phosphorylates IMP1 cotranslationally to promote IGF2 production and the proliferation of mouse embryonic fibroblasts**. *Genes Dev* 2013, **27(3)**:301-312.

34. Estaquier J *et al.*: **The mitochondrial pathways of apoptosis**. *Adv Exp Med Biol* 2012, **942**:157-183.

35. Weinberg F, Chandel NS: **Mitochondrial metabolism and cancer**. *Ann N Y Acad Sci* 2009, **1177**:66-73.

36. Schieke SM *et al.*: **The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity**. *J Biol Chem* 2006, **281(37)**:27643-27652.

37. Bentzinger CF *et al.*: **Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy**. *Cell Metab* 2008, **8(5)**:411-424.

38. Risson V *et al.*: **Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy**. *J Cell Biol* 2009, **187(6)**:859-874.

39. Bentzinger CF *et al.*: **Differential response of skeletal muscles to mTORC1 signaling during atrophy and hypertrophy**. *Skelet Muscle* 2013, **3(1)**:p6.

40. Cunningham JT *et al.*: **mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex**. *Nature* 2007, **450(7170)**:736-740.

41. Romanino K *et al.*: **Myopathy caused by mammalian target of rapamycin complex 1 (mTORC1) inactivation is not reversed by restoring mitochondrial function**. *Proc Natl Acad Sci U S A* 2011, **108(51)**:20808-20813.

42. Goo CK *et al.*: **PTEN/Akt signaling controls mitochondrial respiratory capacity through 4E-BP1**. *PLoS ONE* 2012, **7(9)**:pe45806.

43. Polak P *et al.*: **Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration**. *Cell Metab* 2008, **8(5)**:399-410.

44. • Liu M *et al.*: **Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1**. *Cell Metab* 2014, **19(6)**:967-980.
This study demonstrates that hyperactivation of mTORC1 signaling in adipose tissue leads to obesity and impaired thermogenesis.

45. Hagiwara A *et al.*: **Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c**. *Cell Metab* 2012, **15(5)**:725-738.

46. Yuan M *et al.*: **Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2**. *J Biol Chem* 2012, **287(35)**:29579-29588.

47. Hung CM *et al.*: **Rictor/mTORC2 loss in the Myf5 lineage reprograms brown fat metabolism and protects mice against obesity and metabolic disease**. *Cell Rep* 2014, **8(1)**:256-271.

48. Betz C, Hall MN: **Where is mTOR and what is it doing there?** *J Cell Biol* 2013, **203(4)**:563-574.

49. Sebastian D *et al.*: **Mitofusin 2 (Mfn2) links mitochondrial endoplasmic reticulum function with insulin signaling is essential for normal glucose F homeostasis**. *Proc Natl Acad Sci US A* 2012, **109(14)**:5523-5528.

50. Colombi M *et al.*: **Genome-wide shRNA screen reveals increased mitochondrial dependence upon mTORC2 addiction**. *Oncogene* 2011, **30(13)**:1551-1565.

51. Duvel K *et al.*: **Activation of a metabolic gene regulatory network downstream of mTOR complex 1**. *Mol Cell* 2010, **39(2)**:171-183.

52. • Robitaille AM *et al.*: **Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis**. *Science* 2013, **339(6125)**:1320-1323.
This study together with [53•] demonstrates that mTORC1 stimulates de novo pyrimidine synthesis via S6K-mediated phosphorylation of CAD.

53. • Ben-Sahra I *et al.*: **Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1**. *Science* 2013, **339(6125)**:1323-1328.
This study together with [52•] demonstrates that mTORC1 stimulates de novo pyrimidine synthesis via S6K-mediated phosphorylation of CAD.

54. Rosen ED, Spiegelman BM: **What we talk about when we talk about fat**. *Cell* 2014, **156(1–2)**:20-44.

55. Cybulski N *et al.*: **mTOR complex 2 in adipose tissue negatively controls whole-body growth**. *Proc Natl Acad Sci U S A* 2009, **106(24)**:9902-9907.

56. Kumar A *et al.*: **Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism**. *Diabetes* 2010, **59(6)**:1397-1406.

57. Cornu M, Albert V, Hall MN: **mTOR in aging, metabolism, and cancer**. *Curr Opin Genet Dev* 2013, **23(1)**:53-62.

58. Ricoult SJ, Manning BD: **The multifaceted role of mTORC1 in the control of lipid metabolism**. *EMBO Rep* 2013, **14(3)**:242-251.

59. Lamming DW, Sabatini DM: **A Central role for mTOR in lipid homeostasis**. *Cell Metab* 2013, **18(4)**:465-469.

60. Kenerson HL, Yeh MM, Yeung RS: **Tuberous sclerosis complex-1 deficiency attenuates diet-induced hepatic lipid accumulation**. *PLoS ONE* 2011, **6(3)**:pe18075.

61. Yecies JL *et al.*: **Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways**. *Cell Metab* 2011, **14(1)**:21-32.

62. Sengupta S *et al.*: **mTORC1 controls fasting-induced ketogenesis and its modulation by ageing**. *Nature* 2010, **468(7327)**:1100-1104.

63. Peterson TR *et al.*: **mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway**. *Cell* 2011, **146(3)**:408-420.

64. Umemura A *et al.*: **Liver damage inflammation, and enhanced tumorigenesis after persistent mTORC1 inhibition**. *Cell Metab* 2014, **20(1)**:133-144.

65. •• Cornu M *et al.*: **Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21**. *Proc Natl Acad Sci U S A* 2014, **111(32)**:11592-11599.
This study demonstrates that hyper-activation of hepatic mTORC1 under starvation leads to metabolic stress, thereby affecting whole body physiology.

66. Bookout AL *et al.*: **FGF21 regulates metabolism and circadian behavior by acting on the nervous system**. *Nat Med* 2013, **19(9)**:1147-1152.

67. • Csibi A *et al.*: **The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4**. *Cell* 2013, **153(4)**:840-854.
This study demonstrates that mTORC1 stimulates glutaminolysis.

68. Choo AY *et al.*: **Glucose addiction of TSC null cells is caused by failed mTORC1-dependent balancing of metabolic demand with supply**. *Mol Cell* 2010, **38(4)**:487-499.

69. Newsholme P *et al.*: **Glutamine and glutamate — their central role in cell metabolism and function**. *Cell Biochem Funct* 2003, **21(1)**:1-9.

70. Fischer JE, Chance WT: **Total parenteral nutrition, glutamine, and tumor growth**. *JPEN J Parenter Enteral Nutr* 1990, **14(4 Suppl.)**:86S-89S.

71. Souba WW: **Glutamine and cancer**. *Ann Surg* 1993, **218(6)**:715-728.

72. DeBerardinis RJ *et al.*: **The biology of cancer: metabolic reprogramming fuels cell growth and proliferation**. *Cell Metab* 2008, **7(1)**:11-20.

73. Lamming DW *et al.*: **Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity**. *Science* 2012, **335(6076)**:1638-1643.

74. • Lamming DW *et al.*: **Depletion of Rictor, an essential protein component of mTORC2, decreases male lifespan**. *Aging Cell* 2014, **13(5)**:911-917.
This study demonstrates that ablation of hepatic mTORC2 signaling decreases lifespan of male mice.

75. Egan B, Zierath JR: **Exercise metabolism and the molecular regulation of skeletal muscle adaptation**. *Cell Metab* 2013, **17(2)**:162-184.

76. Yang J: **Enhanced skeletal muscle for effective glucose homeostasis**. *Prog Mol Biol Transl Sci* 2014, **121**:133-163.

77. Mackenzie RW, Elliott BT: **Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes**. *Diabetes Metab Syndr Obes* 2014, **7**:55-64.

78. Lai KM *et al.*: **Conditional activation of Akt in adult skeletal muscle induces rapid hypertrophy**. *Mol Cell Biol* 2004, **24(21)**:9295-9304.

79. Fraenkel M *et al.*: **mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes**. *Diabetes* 2008, **57(4)**:945-957.

80. Houde VP *et al.*: **Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue**. *Diabetes* 2010, **59(6)**:1338-1348.

81. Johnston O *et al.*: **Sirolimus is associated with new-onset diabetes in kidney transplant recipients**. *J Am Soc Nephrol* 2008, **19(7)**:1411-1418.

82. Castets P *et al.*: **Sustained activation of mTORC1 in skeletal muscle inhibits constitutive and starvation-induced autophagy and causes a severe, late-onset myopathy**. *Cell Metab* 2013, **17(5)**:731-744.

83. Kumar A *et al.*: **Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity**. *Mol Cell Biol* 2008, **28(1)**:61-70.

84. Cone RD *et al.*: **The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis**. *Int J Obes Relat Metab Disord* 2001, **25(Suppl. 5)**:S63-S67.

85. Arora S, Anubhuti: **Role of neuropeptides in appetite regulation and obesity — a review**. *Neuropeptides* 2006, **40(6)**:375-401.

86. Neary NM, Goldstone AP, Bloom SR: **Appetite regulation: from the gut to the hypothalamus**. *Clin Endocrinol (Oxf)* 2004, **60(2)**:153-160.

87. Yang SB *et al.*: **Rapamycin ameliorates age-dependent obesity associated with increased mTOR signaling in hypothalamic POMC neurons**. *Neuron* 2012, **75(3)**:425-436.

88. • Kocalis HE *et al.*: **Rictor/mTORC2 facilitates central regulation of energy and glucose homeostasis**. *Mol Metab* 2014, **3(4)**:394-407.
This study demonstrates that inactivation of mTORC2 signaling in POMC neurons leads to obesity.

89. Mori H *et al.*: **Critical role for hypothalamic mTOR activity in energy balance**. *Cell Metab* 2009, **9(4)**:362-374.

90. Matsuzaki H *et al.*: **Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation**. *Proc Natl Acad Sci U S A* 2003, **100(20)**:11285-11290.

91. Iskandar K *et al.*: **PDK-1/FoxO1 pathway in POMC neurons regulates Pomc expression and food intake**. *Am J Physiol Endocrinol Metab* 2010, **298(4)**:E787-E798.

Acknowledgements

I would like to thank Professor Mike Hall for giving me the opportunity to join his research group for my PhD. I am very grateful for his guidance and support that I received throughout the years. I would also like to thank all the past and present members of the Hall Lab, in particular Asami Hagiwara, Marion Cornu and Mitsugu Shimobayashi. I feel very privileged that I had the opportunity to work with these talented scientists.

I would like to thank all core facilities of the Biozentrum, in particular the animal facility and the electron microscopy facility, all collaborators who contributed to the work of this thesis and the Fellowships for Excellence for funding my PhD salary.

Finally, I am grateful for the support I received from my family and friends throughout my PhD. A special thank you goes to Kristoffer Svensson, who always supported and believed in me during all these years. Without him I would not have been able to accomplish any of this.